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THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

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RATIO OF DESOXYPENTOSENUCLEIC ACID TO POTASSIUM IN NORMAL AND MALIGNANT HUMAN TISSUE [1]

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TWO FIGURES

As an outgrowth of the histochemical researches in these laboratories (Lemon and Wisseman, '49) on the acid phosphatases of human malignant tissues as compared with their tissues of origin, further investigations have been planned into the comparative chemistry of such tissues. In this connection, desoxypentosenucleic acid (DNA) is of special interest since it is distributed very similarly to acid phosphomonoesterase. The importance of nucleic acids in relation to the normal growth processes has been amply demonstrateed by the ultraviolet microspectrophotometric technic (Caspersson, '50), while the evidence connecting these compounds with the problem of malignant growth has been excellently reviewed by Stowell ('45).

Nucleic acid concentrations, like enzyme activities, are often measured in terms of total nitrogen content of tissue, assuming the latter to be a measure of the total metabolically active material present. Since tumors and normal epithelial tissues often contain as much as 50% collagen, which is extracellular and metabolically inactive, this assumption leads to large errors in comparative analytical work. An alternative basis for analysis would be the potassium content of tissue. Potassium

is a cellular component, the concentration of which in living cells is approximately 30 times that of extra-cellular fluid and which varies but slightly under all but the most unusual conditions. It can, furthermore, be accurately determined in extremely minute quantities by flame photometry. It was decided, therefore, to determine the DNA/K ratios of representative normal and neoplastic tissue.

METHODS

Tissue sections. While several sensitive methods of analyzing the DNA content of tissues have been reported in the literature (Schneider, '47), these have been uniformly applied, to the best knowledge of the authors, to tissues which have been homogenated, ground or otherwise reduced to near-molecular dispersion. There are obvious advantages, however, to the direct analysis of tissue sections. In the first place, the slicing of fresh, rapidly-frozen specimens with a freezing microtome alters but slightly the enzymatic and morphologic composition of the material, so that nucleic acid determinations can be conducted directly on tissue which has previously been used for enzymatic studies. Secondly, the use of slices permits the inclusion of histological controls, which are still all too frequently neglected in biochemical analyses of non-homogeneous malignant tissues, and the preparations of multiple tissue aliquots based upon statistically valid sampling of the specimen.

To prepare the slices, human tissue, removed at operation, is cut into blocks of 1 or 2 cm in length and a few millimeters wide and thick. These are placed in small test tubes and rapidly frozen in a mixture of solid carbon dioxide and acetone, after which they are placed in a deep freeze unit maintained at $-70°C$. Just prior to chemical analysis, the tissue-block is sliced into sections of 25 µ thickness by means of a freezing microtome. Alternate sections are serially distributed into tubes for DNA, K, N and other analyses until 4 to 8 sections are present in each tube. Usually the tissue

aliquots contain between 0.1 and 0.2 mg of nitrogen, when duplicate aliquots are analyzed by the micro-Kjeldahl method of Wong ('23).

Standards. Sperm desoxypentosenucleic acid, obtained from several commercial sources, was used as a standard. Its phosphorus content, as analyzed in this laboratory by the method of Fiske and Subbarow ('25) was found to be 9.7% as compared with a theoretical 9.9% for the tetranucleotide, $C_{39}H_{51}N_{15}P_4O_{25}$. In preparing the DNA standard, 50 mg of the compound was dissolved in 35 ml of 0.1 NaOH and the solution made up to 100 ml.

Potassium content. These sections should be passed directly from knife to test tube, using a dissecting needle. One-tenth milliliter concentrated nitric acid is added and the test tube is placed in the icebox overnight, after which it is heated in a boiling water bath for one hour. The oxidized products are filtered, 20 ml of 0.05 N nitric acid being used as a wash. An internal lithium standard is added when making the solutions up to 50 ml, and the potassium content is then determined by a Perkin-Elmer flame photometer.[2]

DNA content. The analysis of nucleic acids may be carried out either by the determination of inorganic phosphate ion liberated under specified conditions or by the determination of the sugar constituent. Methods of analysis by phosphate liberation have been successfully applied (Schmidt and Thannhauser, '45; Schneider, '46) but are less useful for our purposes than are sugar analyses since it is frequently desirable to assay consecutively the phosphatase and DNA content of the same tissue specimens. In that case the phosphatase analyses would introduce large quantities of extraneous phosphate ion into the system.

The analytical methods for DNA which involve the colorimetric determination of the sugar constituent may be listed according to the organic reagent used. These are tryptophane

[2] This method was suggested by Dr. W. M. Wallace, Children's Hospital, Boston.

(Cohen, '44), diphenylamine (Dische, '30), carbazole (Dische, '30), cysteine (Dische, '44; Stumpf, '47), phloroglucinol (von Euler and Hahn, '47; Villela, '49), and sulfite-decolorized fuchsin (Feulgen and Rossenbeck, '24). Of these, the last, commonly known as the Feulgen reagent, is primarily employed in the histochemical location of DNA and is not readily adaptable to quantitative colorimetric analysis, although current attempts in that direction are being made (DiStefano, '48; Ris and Mirsky, '49). The carbazole method has been modified and investigated (Gurin and Hood, '41) and found to be effective only where rigidly purified reagents were used and in the absence of a variety of possibly interfering substances. It is consequently not easily applicable to the rapid routine analyses contemplated here.

In the case of the cysteine and phloroglucinol methods, the colors formed are not stable, varying markedly and continuously for at least an hour after formation, thus rendering quantitative work difficult (fig. 1). None of the above-named objections apply either to the diphenylamine or to the tryptophane methods, and these two were therefore studied intensively. The experimental details of the two methods are included in table 1. In the tryptophane method, the color intensity is read at 480 mµ. Cohen ('44) uses 520 mµ instead, but the absorption spectrum he reports indicates the peak to be at about 500, and our own curves place it at 480. In using this method, the tissue slices are first washed with 5 ml isotonic NaCl solution for 5 minutes in order to remove pentose-nucleic acid (see comment by H. Ris in Cohen, '47) which would otherwise interfere in the determinations. The diphenylamine method was carried out as described by Seibert ('40). In order to adapt the latter method to tissue work, the slices to be analyzed were first treated with trichloracetic acid (Schneider, '45) in order to extract DNA from the cellular proteins.

The standard curves which are obtained for both the diphenylamine and the tryptophane methods are linear up to

total DNA contents of at least 250 µg. From the slope of these curves, the final volumes of the colored complexes, and the diameter of the cuvettes used in each case, the relative molar extinctions (which are a measure of the comparative potential sensitivities) may be obtained. This information is presented
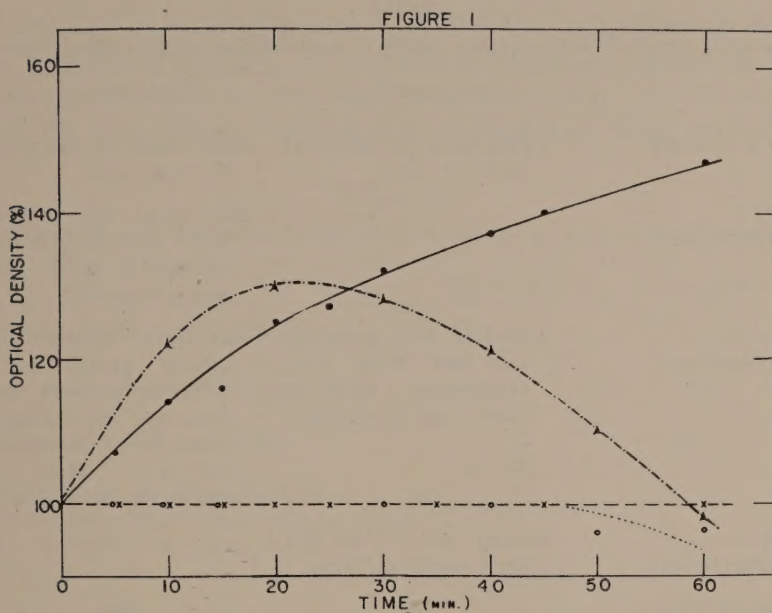
FIGURE I

Fig. 1   The stability of the color-complex resulting from the treatment of DNA by the diphenylamine method (– – × – – – – ×); the tryptophane method (...○....○..); the cysteine method (—●——●—); and the phloroglucinol method (—.△.—△.—). A 5-minute period after completion of each reaction is allowed for the development of color before optical density measurements are made. In each case, the optical density measurement after the 5-minute waiting period is set equal to 100, all other readings being converted to per cent of original.

in concise form (table 2). It will be noted that the diphenyla-mine method is over twice as sensitive as is the tryptophane method.

To test the effect of protein upon each of the methods be-ing considered, solutions of egg albumin and of edestin (rea-

gent grade and presumably DNA-free) were tested in varying amounts by each of the methods (fig. 2). It will be seen that whereas the diphenylamine method gave virtually no reaction up to quantities of protein 3 to 5 times as high as might be expected in tissue analyses, significant interference was ap-

TABLE 1

Outline of analytical methods tested for the determination of DNA in tissue sections

	TRYPTOPHANE	DIPHENYLAMINE
To be analyzed	Tissue slices (or standard) in 0.4 ml water.	Tissue slices (or standard) in 0.5 ml water.
Step 1 (Extraction)	. . . .	Add 2.5 ml 5% trichloracetic acid. Heat for 15 minutes at 90°C. Discard precipitate.
Step 2 (Digestion)	Add 1 ml 60% perchloric acid and 0.3 ml 0.5% tryptophane. Heat at 100°C. for 30 minutes.	Add 6 ml of mixture containing glacial acetic acid, concentrated sulfuric acid and diphenylamine in the proportions 100 : 2⅔ : 1. Heat at 100°C. for 10 minutes.
Step 3 (Extraction)	Extract with 1.7 ml isoamyl alcohol and filter.	. . . .
Color of DNA complex	Pink	Blue
Absorption peak of complex	480 mμ	600 mμ
Colorimeter used	Coleman junior spectrophotometer	Evelyn photoelectric colorimeter

parent in the case of the tryptophane method. Von Euler and Hahn ('47) state that proteins do interfere with the diphenylamine reaction, but it is not certain from their report that they used the preliminary trichloracetic acid extraction for a quantitative separation of DNA and proteins before initiating the color-forming reaction.

In comparing the tryptophane and diphenylamine methods in actual tissue experiments, serial sections of a given tissue block were placed singly in 12 or more test tubes. The process was repeated until each tube contained about 5 slices. In this manner, histological variations among the samples were minimized. Half the tubes, taken at random, were then analyzed by the tryptophane method and the remainder by the diphenylamine method. The results obtained are contained in table 3. As would be expected from the protein interference with the tryptophane method, that method was found to yield

TABLE 2

Sensitivity of analytical methods used in determination of DNA in tissue sections

	TRYPTOPHANE	DIPHENYLAMINE
Optical density per 100 μg DNA	0.065	0.074
Final volume of colored complex (ml)	1.7	9.0
Diameter of cuvette used (mm)	7	20
Optical density per 100 μg DNA at equal light paths and final volumes. (That for tryptophane set equal to 1.00.)	1.00	2.11

consistently higher results, the ratio of ''Tryptophane DNA''/''Diphenylamine DNA'' averaging 1.55 over all 5 cases. (In 26 routine analyses conducted on duplicate tissue samples, both normal and neoplastic, by each method, the corresponding ratio was 1.82.) A specific interference with the tryptophane method seemed to be present in the case of kidney tissue, where a yellow to orange color, masking the normal clear pink was obtained. Results were obviously abnormal, being far higher than those for any other tissue examined. The DNA content of kidney tissue as determined by the diphenylamine method, however, was normal as compared with the other tissues tested.

Since the diphenylamine method would thus seem to be superior to the tryptophane method both in sensitivity and specificity, it was the method of choice for the analysis of tissue sections for DNA.
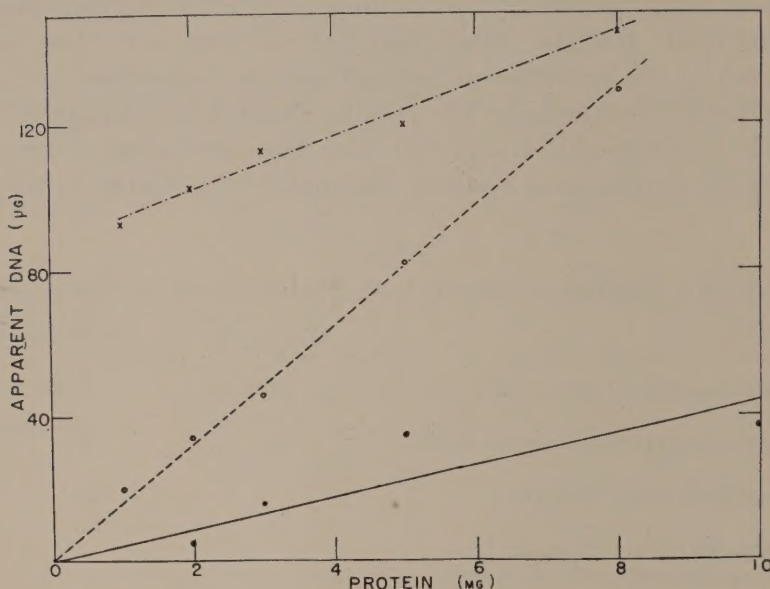

Fig. 2   The color formation resulting from the analysis of egg elbumin by the diphenylamine method (—●——●—), and by the tryptophane method (——O———O—); and from the analysis of edestin by the tryptophane method (— × —.—. ×). The intensity of color in all cases is converted into "apparent DNA" using the conversion factors appropriate for the method (see table 1). The analysis of edestin by the diphenylamine method results in no detectable color formation up to 10 mg of protein.

RESULTS AND DISCUSSION

Table 4 includes data obtained on the DNA content per unit potassium of 5 representative human tissues. From the figures, two conclusions may be drawn. First, the values obtained cluster strongly in the range 0.10–0.26, only 5 of the 27 readings falling outside those limits. Second, there would seem to be no definite trend whereby the ratio in cancerous tissue can be differentiated from that in the normal tissue of

TABLE 3

DNA content of representative tissue samples as determined by the tryptophane and diphenylamine methods

NATURE OF TISSUE	1. GASTRIC MUCOSA		2. KIDNEY		3. GASTRIC MUCOSA		4. APPENDIX		5. BREAST (FIBROADENOMA)	
Method used	Trypto-phane	Diphenyl-amine	Trypto-phane	Diphenyl-amine	Trypto-phane	Diphenyl-amine	Trypto-phane	Diphenyl-amine	Trypto-phane	Diphenyl-amine
Optical densities readings on tissue slices	.086	.052	.104	.078	.079	.071	.047	.066	.053	.046
	.070	.047	.118	.064	.099	.085	.060	.072	.041	.046
	.077	.061	.184	.066	.077	.072	.059	.069	.073	.048
	.088	.037	.110	.068	.090	.064	.056	.071	.061	.072
	.069	.020	.131	.059	.086	.071	.052	.063	.062	.058
	.073	.051	.139		.060	.057	.066	.068	.061	.051
									.058	.042
Mean optical density	.0772	.0447	.1310	.0670	.0818	.0700	.0567	.0682	.0584	.0519
Average deviation of a single observation from the mean (a.d.) and per cent a.d.	.0066 (8.5%)	.0108 (24.2%)	.0202 (15.4%)	.0040 (6.0%)	.0098 (12.0%)	.0063 (9.0%)	.0050 (8.8%)	.0015 (2.2%)	.0066 (11.3%)	.0075 (14.5%)
DNA content (μg)	119.0	60.4	202.0	90.5	126.0	94.6	87.2	92.1	89.8	70.1
Ratio $\dfrac{\text{Tryptophane ``DNA''}}{\text{Diphenylamine ``DNA''}}$	1.97		2.23		1.33		0.95		1.28	

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TABLE 4

DNA/K ratios of normal and cancerous tissues

TISSUE	TOTAL DNA	TOTAL K	AVERAGE DNA/K
	mg	$\mu Eq.$	$mg/\mu Eq.$
Stomach (normal)	0.157, 0.117	0.55, 0.90	0.19
	0.166, 0.211	0.95, 1.10	0.18
	0.136, 0.111, 0.134, 0.111	0.80	0.15
	0.119, 0.131, 0.098	0.65	0.18
	0.051, 0.045	0.20, 0.20	0.24
Stomach (cancer)	0.157, 0.136	0.60, 0.45	0.28
	0.162, 0.157	0.60, 0.50	0.29
Rectum (normal)	0.102, 0.096	0.55, 0.40	0.21
	0.068, 0.070	0.30, 0.25	0.25
	0.126, 0.155	0.85, 0.70	0.18
Rectum (cancer)	0.145, 0.126	0.60, 0.75	0.20
	0.098, 0.100	0.60, 0.55	0.17
	0.070, 0.091	0.40, 0.70	0.15
Cervix (normal	*0.064, 0.051	0.15, 0.30	0.26
	*0.051, 0.034	0.20, 0.25	0.19
	0.064, 0.051	0.45, 0.50	0.12
Cervix (cancer)	0.070, 0.100	0.30, 0.35	0.26
	0.113, 0.102	0.40, 0.50	0.24
	0.072, 0.081	0.60, 0.50	0.14
Kidney (normal)	0.155, 0.115	1.00, 0.75	0.15
	0.074, 0.079	0.55, 0.65	0.13
	0.091, 0.094	0.90, 0.90	0.10
Kidney (cancer)	0.100, 0.111	1.60, 1.05	0.08
	0.045, 0.036	0.80, 0.85	0.05
	0.113, 0.110	2.00, 1.30	0.07
Colon (normal)	0.102, 0.045	0.30, 0.35	0.22
Colon (cancer)	0.098, 0.070	0.70, 0.70	0.12

Average DNA/K of all normal tissue samples 0.183
Average DNA/K of all cancerous tissue samples 0.171

* Some tumor tissue present.

homologous origin. Such results would be consistent with the view that the concentration of DNA per unit mass of living matter tends to be constant, and that this concentration is not affected by the neoplastic change.

If, then, there is a difference in the DNA of cancerous tissue as opposed to that of normal tissue (and such a hypothesis, in view of the importance of nucleic acids in heredity and growth, is most attractive), it may not be merely a quantitative one. The difference instead may be one of structure and it is in that direction that investigations in these laboratories are now tending.

Stowell ('46) has determined the DNA content of individual cells, both normal and cancerous, by microphotometric measurements of the intensity of a Feulgen stain. He reported increases in the DNA contents of cancerous cells as compared with normal ones, and also increases in the DNA content per unit volume. However, such increases might well have reflected increase in cell mass or in concentration of metabolically active material rather than increase of DNA concentration. The use of DNA/K ratios in this investigation minimizes the possibility of such variations.

SUMMARY

Concentrations of desoxypentosenucleic acid in repesentative human tissues, including both cancerous tissue and the normal tissue of homologous origin, were determined by the Dische diphenylamine method. The diphenylamine method was shown to be the method of preference for application to the direct determination of DNA in intact tissue sections, since the chief alternate method considered, the Cohen tryptophane method, was less sensitive and specific. Potassium analyses by the flame photometer were used as a measure of total metabolizing tissue present.

The DNA/K ratios were found to be relatively constant in the tissues tested and to show no marked difference in the cancerous as opposed to the normal tissues. It is suggested

that differences in the DNA of cancerous and normal tissues, if they exist, may be not of quantity merely, but more probably involve actual changes in structure.

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THE BIOSYNTHESIS OF STABLE COBALTO-PROTEINS BY PLANTS [1,2]

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SEVEN FIGURES

The role of cobalt as an essential element in metabolism and nutrition has been the focus of considerable investigation recently with the discovery that certain diseases of sheep and other ruminants occur on pasturages in which the cobalt concentration is low.[3] Remission of the symptoms occurs if cobalt is added to the diet, thus demonstrating that cobalt is an essential element for these animals. So far, however, it has not been possible to obtain a cobalt deficiency in animals other than the ruminant, nor is there evidence for the essentiality of cobalt in green plants.[3] Recently the isolation of vitamin B_{12} and the demonstration that this vitamin contains cobalt as an integral part of its structure (Smith, '48; Rickes, Brink, Koniuszy, Wood and Folkers, '48), have made it necessary to reexamine the essential nature of cobalt in metabolic systems. It is well known that vitamin B_{12} forms a part of the anti-pernicious anemia complex (West, '48; Spies, Stone, Garcia Lopez, Milanes, Lopez Toca, and Aramburu, '48) and since some bacteria also require vitamin B_{12} as a growth factor (Shorb, '48), one is led to suspect that this element may be ubiquitously distributed in nature as an integral part of metabolizing systems. In addition to

cobalt being metabolically involved as a constitutent of the vitamin B_{12}, there is also the recent observation of Smith ('48) on the activation of intestinal peptidases by the cobalt ion. He showed that the degree of chelation of a substrate with the cobalt ion correlates with the relative rate of splitting of the various substrates by the enzyme system. This information taken as a whole would indicate that cobalt forms an essential part of metabolizing systems not only as a constitutent of vitamin B_{12}, but possibly also as a part of enzyme systems in the cell.

EXPERIMENTAL

A. *Culture methods.* The majority of the experiments to be reported below have been carried out with the wild-type strain 5297A of *Neurospora crassa*, grown on a modified Fries medium [4] Cobalt-60 as cobaltous chloride, diluted with sufficient carrier to give the desired cobalt concentration, was added to the medium. The *Neurospora* was grown at 30°C. in 1 l Erlenmeyer flasks containing 250 ml of the Fries medium. The flasks were shaken twice a day. Since the cobalt-60 has a half-life of 5.3 years, and since each experiment extended for not more than two months, no correction for radioactive decay was made.

B. *Radioactive assay.* A sample of approximately 100 mg of organic matter (fresh, dried or homogenized mycelium) was placed in 125 ml Erlenmeyer flask together with 1 mg cobalt carrier. It was ashed by boiling with 4 ml sulfuric acid and sufficient nitric acid to produce a clear digest after reducing to white fumes. The sample was cooled and diluted to approximately 50 ml with water, after which it was neutralized to phenolphthalein with pellet sodium hydroxide. The solution was boiled while three 50 mg portions of sodium perborate were added to oxidize the cobaltous hydroxide

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[4] The Medium of Ryan, Beadle and Tatum ('43) was modified by the omission of ammonium tartrate, following the observation that little added growth was obtained in its presence. Since the tartrate ion is a chelating agent for some metals, it might reduce the effective concentration of ionic cobalt present.

to the cobaltic state. The cobaltic hydroxide was filtered on a sintered glass Gooch crucible of medium porosity, and washed with a little water containing 0.1% (W/V) Aerosol OT.[5] The crucibles were those designed for use with the proportional counter of Bernstein and Ballentine ('49). The sample prepared for counting was a uniform coat on the sintered surface. Since 1 mg of carrier was uniformly added to each sample before ashing and since the amount of cobalt present in the analytical sample itself was extremely small, the amount of precipitate did not significantly vary between individual analyses; consequently, no correction for self-absorption was made, this being incorporated in the overall geometry of the counting system. The geometry for counting of the 0.32 mev beta-rays from cobalt-60 was 40%. The standard error of replicate samples carried through the complete procedure was $\pm$ 2.35%.

In addition to the above procedure, we also employed the precipitation of cobalt as cobaltous sulfide. The ashing procedure was the same. The acid digest was neutralized to methyl orange with sodium hydroxide. One and one-half milliliters of pyridine (Ostroumov, '38) was added to the boiling solution which was then saturated with hydrogen sulfide. The precipitate of cobalt sulfide was filtered on the Gooch crucible, and washed with a 0.1% (W/V) solution of Aerosol OT. After drying several hours in an 80° oven, the cobalt sulfide was coated with a thin layer of collodion formed from a 1% solution of (W/V) Mallinckrodt Parlodion in acetone. From the time of sample preparation until being counted, the crucibles were either allowed to remain in the drying oven or were kept in a desiccator over calcium chloride. This procedure utilizing cobalt sulfide, while giving a better appearing sample, was employed only for those solutions containing high concentrations of the alkaline earths which interfered in the hydroxide precipitation method. This was due to the fact that minute traces of water led to pro-

[5] Aerosol OT, 100% pellets, Product of American Cyanamid Company.

found drifts in the counting rate, and it was very difficult to stabilize the counter when a sulfide containing precipitate was present in the counting chamber. The reasons for this instability of the counter in the presence of sulfide is being further investigated. In the use of the methane-flow proportional counter it is advisable to introduce a drying-tube containing magnesium perchlorate in the counting gas line to insure absolute dryness of the counting gas.

The results in this paper are expressed in terms of micromoles of cobalt present. This value was obtained from the number of counts per micromole of cobalt as obtained from Oak Ridge.[6] A simple proportionality permits the conversion from the counts per minute in the sample to micromoles of cobalt present.

C. *Homogenization and protein assay.* A wide variety of methods have been investigated in our laboratory for preparing homogenates of *Neurospora* so as to contain a maximum concentration of soluble protein. We have tried lyophilization followed by dry grinding, grinding of wet mycelium with sand, ball mill grinding of both lyophilized and wet mycelium and chopping in a Waring Blendor at 0°C. for various lengths of time. None of these methods gave a satisfactory extraction of protein from the mycelium. Two methods have proved to be extremely useful. One is the grinding of the mycelium frozen with liquid nitrogen in a mortar. The other method is grinding in the Ten Brock homogenizer.[7] Both these methods give a liberation of soluble protein closely approximating the maximum yield, such as may be obtained by homogenization of the mycelium with 1 N sodium hydroxide. A variety of extracting solutions were tested, keeping in mind the desirability of extraction of the protein without the risk of drastic denaturation. Various concentrations of sodium chloride, and various pH's were tested; the optimum concentration is 1% sodium chloride

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[6] The cobalt-60 solution H 19 used in this investigation was supplied by the Isotopes Division, U. S. Atomic Energy Commission.

[7] Scientific Glass Apparatus Company, Bloomfield, New Jersey.

containing M/15 phosphate buffer at pH 7.4 at a ratio of from 5:1 to 10:1 buffered salt to mycelium. Under these conditions we believe that nearly quantitative extraction of soluble protein from the mycelium is possible, provided careful grinding at low temperature is carried out for a sufficient period.[8]

The amount of protein extracted was determined by a modified biuret method (Robinson and Hogden, '40), determining the color formed in the Model B Beckman Spectrophotometer at 565 mµ. The method was calibrated in terms of biuret color produced from a sample of 5 times recrystallized and electrodialyzed egg albumen.

Dry weights of homogenate and mycelium were obtained by drying overnight at 80° in an oven. Correction for the weight of dissolved salt was made in the case of homogenate samples.

To determine total soluble protein in the mycelium the following analyses were carried out: (1) The mycelium was homogenized and a sample of the whole homogenate was taken for the estimation of the total radioactive cobalt present. (2) An aliquot of the homogenate was centrifuged for 35 minutes in the Servall Superspeed centrifuge at 16,000 g. This force was sufficient to bring down all of the cell debris, probably all the nuclei and mitochrondia but left the "microsomes" in suspension. On this sample both the radio-cobalt and the total protein were estimated by the appropriate methods. (3) To an aliquot of this centrifuged homogenate, the sodium salt of ethylenediamine tetracetic acid (Versene) was added to give a concentration of 0.1%. This aliquot was then dialyzed[9] against running tap water for 24 hours in a rocking dialyzer. At the end of this time the samples were removed from the dialysis sack and diluted

[8] The grinder was driven with a slow speed motor at 200 r.p.m., coupled through a loose rubber connection which would slip in case the homogenizer jammed. The mycelium was ground in ca. 3 gm portions, each portion requiring about 2–5 minutes of grinding.

to a known volume with the buffered salt solution. Duplicate aliquots of the diluted, dialyzed homogenate were then centrifuged in the Servall at 16,000 g for two to three hours. This procedure sufficed to separate the "microsomes" from the solution. Radioactive assays were carried out on both the supernatant and the sedimented "microsomes."

After dialysis with Versene the cobalt remaining is designated as stably-bound cobalt. That the procedure of dialysis with Versene sufficed to remove any ionically-bound cobalt or free ionic cobalt was demonstrated by the following expriment. To a homogenate of *Neurospora* grown in the absence of radioactive cobalt, a sample of radio-cobalt was added well in excess of the amount normally found in the "tracer" homogenates. This solution was then subjected to a variety of treatments. Aliquots were dialyzed against running water (a) without any additive, (b) with carrier cobalt added to the level of about 10^7 times the tracer cobalt present, (c) without carrier but with the addition of Versene to the level of 0.1%. In table 1 the results of three such experiments are tabulated, showing that dialysis for 24 hours with Versene added was sufficient to remove 98% of the contaminating radioactivity.

Similar removal of contaminating radioactive cobalt added to "cold" homogenate can be achieved by precipitation with trichloracetic acid either in the presence of large amounts of carrier or of Versene. When *Neurospora* is grown in the presence of radioactive cobalt tracer, the trichloracetic acid precipitate contains radioactivity many times that in the radio-chemical control.

RESULTS AND DISCUSSION

The data presented in table 1 show that when tracer cobalt is added to *Neurospora* homogenates it may be quantitatively removed by dialysis in the presence of ethylenediamine

⁹ Cellophane tubing, ¾″, sold by Fisher Scientific Company, Pittsburgh, Pennsylvania.

tetracetic acid (Versene). When a similar amount of cobalt is present in the homogenate as the result of accumulation during growth, a considerable fraction of this cobalt is bound

TABLE 1

Radiochemical control

Comparisons of radioactivity remaining in Neurospora mycelial homogenates when cobalt-60 is added either before or after growth. Dialysis against running tap water. Versene was added to a final concentration of 0.1%; carrier cobalt was added at > 10⁶ times the concentration of tracer cobalt

TREATMENT	% INITIAL COUNTS REMAINING	
	Co^{60} after growth	Co^{60} during growth
I		
None	(4,760 cpm/gm WW)	(4,930 cpm/gm WW)
Static dialysis only, 42 hrs.	15.0	. . . .
Static dialysis with carrier added	4.4	33.8
Static dialysis with Versene	2.3	28.3
II		
None	(8,500 cpm/gm WW)	(5,500 cpm/gm WW)
Rocking dialysis only, 24 hrs.	5.4	. . . .
Rocking dialysis with Versene added	1.7	37
III		
None	(18,800 cpm/gm WW)	(15,800 cpm/gm WW)
Rocking dialysis, 24 hrs.	4.9	24.5
Rocking dialysis with Versene added	2.1	19.5
Rocking dialysis with carrier added	3.3	18.5
Trichloracetic acid precipitated,	5.0	17.1
with Versene added	3.3	17.0
with carrier added	3.8	17.0

so that a similar dialysis with Versene does not remove it. This cobalt does not dialyze through a cellophane membrane, it is precipitated by trichloracetic acid, and it does not exchange with ionic cobalt. For these reasons this cobalt is believed to be stably bound by metabolic processes as cobalto-

protein. We have investigated the amount and rate of forma-
tion of this cobalto-protein during the growth phases of the
mold *Neurospora*.

A. *Growth and protein formation in Neurospora.* In fig-
ure 1 the data are presented for both the rate of formation
of mycelial mass as well as for the formation of total soluble
protein. The value for *total* soluble protein is obtained by
making certain assumptions and corrections. The base value
is the protein content of the clarified, centrifuged homogenate,
determined by the biuret method. From our radio-cobalt
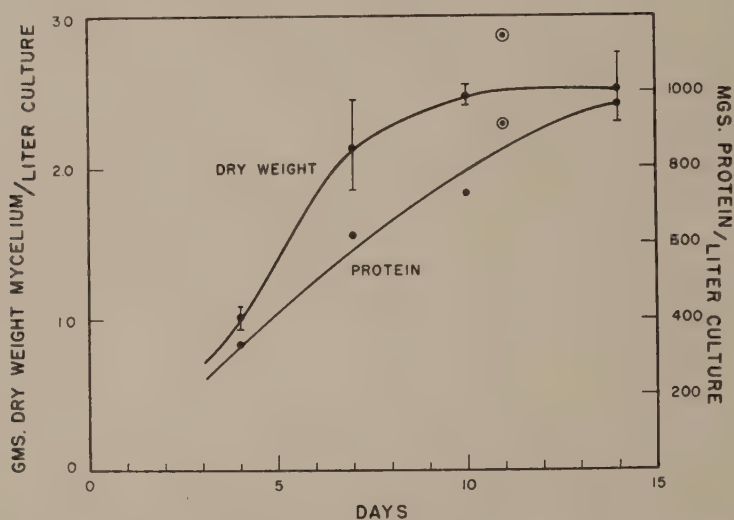
Fig. 1 *Neurospora* growth and soluble protein synthesis. ● are averages of
4 values except ⊙ which are single determinations.

determinations the ratio of cobalt present in the intact my-
celium to the amount extracted into the homogenate is known.
Numerous experiments indicate that by very careful homoge-
nization all the cobalt will be present in the homogenate,
which would be the case if it is present either as ionic cobalt
free in the cytoplasm or as cobalto-protein soluble under the
conditions of extraction. It is not practical to routinely
achieve such complete extraction of the cobalt and protein.

However, if all the cobalt is extractable, then the ratio of cobalt in the mycelium to that in the homogenate provides a direct estimate of the efficiency of extraction, and may be employed as a correction factor. In table 2 data are presented for three experiments in which the amount of homogenization was deliberately varied, the mycelium being obtained from *Neurospora* grown with tracer cobalt. In column 1 the base

TABLE 2

Estimation of total soluble protein in mycelium

In each experiment aliquots of mycelium were subjected to various degrees of homogenization. (1) The milligrams protein found in the centrifuged homogenate is referred to the amount (dry weight basis) of mycelium taken. (2) The per cent efficiency of homogenization is given by the ratio of radioactive cobalt in the initial aliquot of mycelium to that in the homogenate. (3) The soluble protein content of the mycelium is obtained by correcting the values of (1) by the efficiency of homogenization (2)

	(1) MG P/100 MG DRY WT. MYCELIUM	(2) EFFICIENCY OF HOMOGENIZATION	(3) CORR. PROTEIN MG P/100 MG DRY WT.
Exp. I – A	21.0	47.3%	44.5
B	25.3	52.5	48.2
C	28.9	60.5	47.8
Av.			46.8 ± 2.03
Exp. II – A	11.1	42.1%	26.1
B	25.7	83.8	31.0
C	23.9	84.6	28.4
Av.			28.5 ± 2.45
Exp. III – A	19.5	62.0	31.5
B	23.5	66.7	35.2
C	28.8	91.2	31.7
Av.			32.8 ± 2.08

value for protein extracted is given as milligram protein per 100 mg dry mycelium. As would be expected these values scatter widely for identical amounts of mycelium subjected to varying degrees of homogenization. The efficiency of homogenization is given in column 2 as estimated by the above mentioned cobalt ratio. When this value for efficiency is used as a correction factor, it is found that the amount of

protein estimated in the whole mycelium is a constant within
the analytical errors. Therefore as a first approximation
this correction is assumed to be valid, and is applied to our
data both for total protein and for *stable* cobalt in the my-
celium.

Comparison (fig. 1) of the mycelium weight curve with
that of protein formed, which comparison is more graphically
made in figure 2, shows that growth in the sense of mass
increase is not strictly paralleled by the synthetic processes
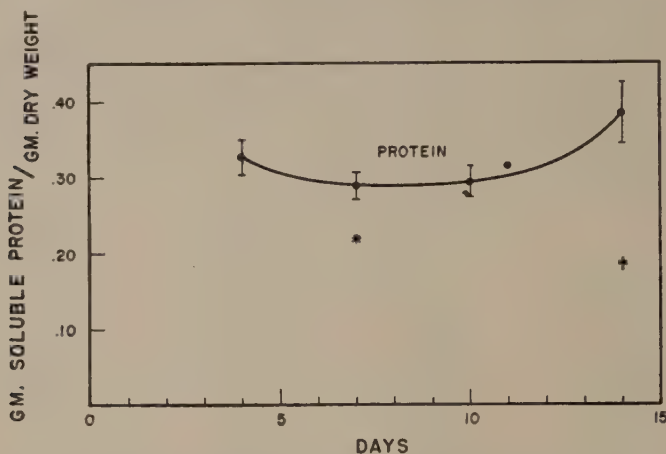
Fig. 2   Soluble protein content of *Neurospora* mycelium. * and ‡ omitted from
averages; others are the average of 4 values.

involved in the formation of soluble protein. Indeed we find
protein synthesis proceeding at a time when weight increase
has ceased. In experiments to be reported in a subsequent
publication, an even higher degree of disjunction of increase
in mycelial mass and increase in protein content has been
achieved by alteration in physiological conditions, such as
oxygen tensions.

One may also point out with respect to the data presented
in figure 1, that added cobalt in the concentrations we used
does not affect the growth, either by inhibition or by stimu-
lation.

B. *Accumulation of cobalt by Neurospora.* We have investigated the accumulation of total cobalt by *Neurospora* for concentrations of this ion varying by a thousand-fold. The results are presented in figure 3. Similar to the absence of a strict correlation between mass increase and protein
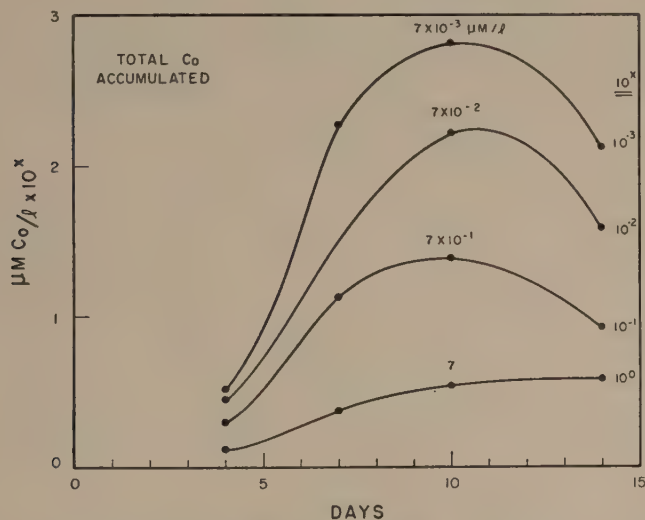
Fig. 3 Total cobalt accumulation in *Neurospora* mycelium. Ordinate: μM total cobalt accumulated b ythe mycelium formed in 1 l modified Fries medium. The ordinate scale factor, 10^x, is given at the right, permitting data for 4 cobalt concentrations to be presented on the same graph. The number above each curve gives the cobalt concentration fed in the medium.

Abscissa: Days of growth at $30°C$. in standing cultures, 250 ml medium per liter flask.

synthesis, the pattern of cobalt accumulation follows in only a general way the growth pattern of the cultures (refer to figs. 2 and 3). Early in the cultural history we find protein synthesis leading the mass increase while very little cobalt is being accumulated. When optimum growth is reached, a similar optimum in cobalt uptake has occured while the protein synthesis has fallen behind. Finally in old cultures the mass remains static or decreases slightly, sizable losses in cobalt occur, but a relatively rapid rate of protein synthesis con-

tinues. Studies on changes in culture metabolism which might correlate with these changes, especially the rate of cobalt uptake, are now under investigation.

An interesting point is the efficiency of accumulation of cobalt by the mycelium. Working with *Neurospora crassa,* Andersson-Kottö and Hevesy ('49) concluded that this organism accumulated zinc far more efficiently than cobalt. However they used a relatively high concentration of cobalt, $3 \mu M/L$, as compared to our lowest concentration, 7×10^{-3}
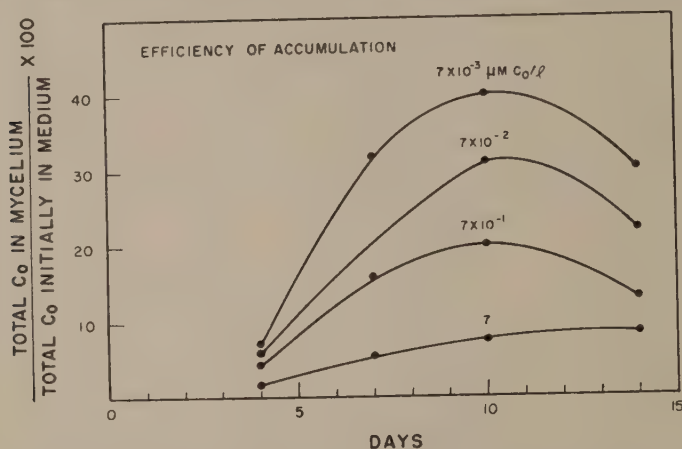
Fig. 4 Efficiency of cobalt accumulation by *Neurospora.* The percent cobalt fed that is present in the mold mycelium at various times of growth. The concentration of cobalt fed is given above each curve.

$\mu M/L$. With a comparable concentration of $7 \mu M/L$ we also find a low efficiency of accumulation as shown in figure 4, when only 1–10% of the cobalt is removed from the medium depending on the length of growth. At the lowest concentration, however, over 40% of the fed cobalt is removed after 10 days which suggests that there is a mechanism for cobalt concentration. That this accumulation occurs against a concentration gradient is seen from the curves of figure 5 giving the *concentration ratio* for cobalt in the medium to that in the mycelium. A maximum for our experiments is a 23-fold increase in cobalt concentration. Such a

concentration of ions of the trace metals is a common and well-known phenomenon. Many green plants will accumulate ions to a concentration of several thousand times that occurring in the soils on which they are grown. Such a concentration effect does not imply that the element is an essential one.

C. *Formation of cobalto-proteins by Neurospora.* In view of the findings presented in table 1, our primary interest
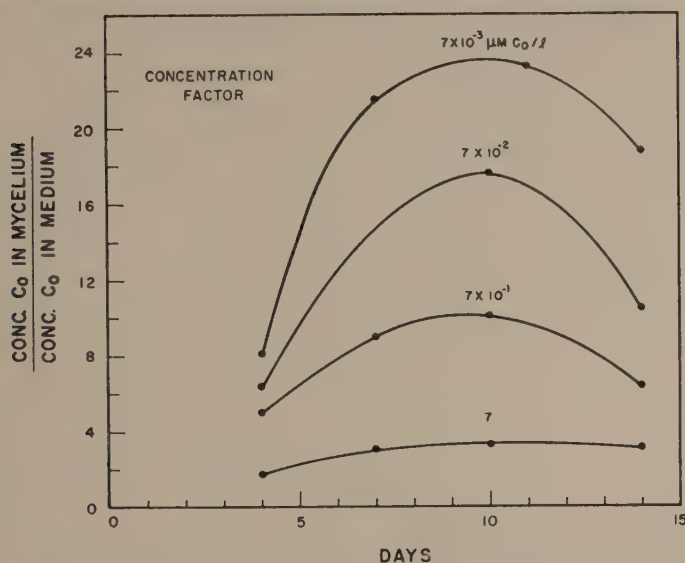
Fig. 5 Concentration of cobalt by *Neurospora.* The ratio of cobalt present in a gram of wet mycelium compared to the cobalt remaining in a cubic centimeter of medium at various cobalt concentrations.

is in the amount of cobalt incorporated in stably-bound form during the growth of the mold mycelium. The data obtained are presented graphically in figure 6, which roughly parallels that for total cobalt accumulation. The quantitative relationship between *stable* and *total* cobalt is shown in figure 7. It is difficult to judge how much these results reflect normal metabolic processes in distinction to those induced by an abnormally high cobalt ion concentration. Actually at a concentration of 7×10^{-3} μM/L and 10 days growth, there are

only about 10^{-5} µg of cobalt per gram dry mycelium. While this may be considerably in excess (we estimate not more than 10-fold) of the cobalt introduced by contamination of salts, nutrients, water and glassware, it would still seem to be within a possible physiological range.

We have been able to further fractionate the stable cobalt. The centrifuged homogenate (16,000 g for 30 minutes) is extremely turbid due to particles of nearly sub-microscopic size. These particles are not removed by filtering with
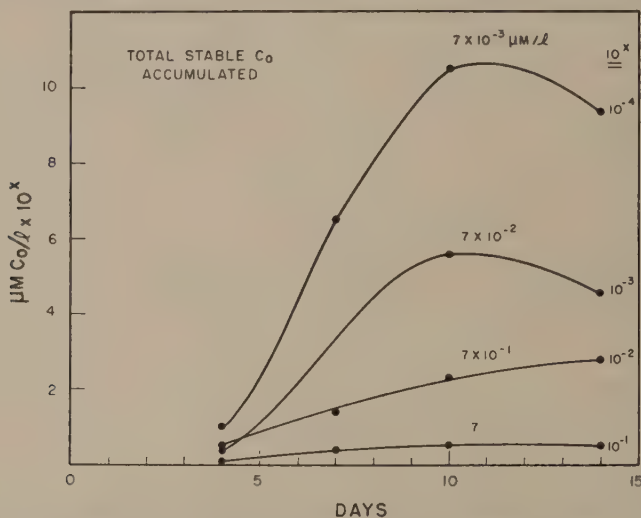
Fig. 6 Total stably-bound cobalt accumulation in *Neurospora* mycelium. *Stable* rather than *Total*, but otherwise same as figure 3.

John Manville Hyflow Celite (1% W/v), and none of the cobalt is lost by this procedure. Several hours of centrifuging at 16,000 g will harvest this particulate component, and permit the resolution of the stable cobalt into two fractions — the particulate and the soluble components. Under the conditions of growth employed in this investigation there is no significant variation of the relative proportions of *stable* cobalt found in these two fractions with respect to either cobalt ion concentration or to the time of growth. The soluble fraction contains 57.4% of the stably-bound cobalt. Whether

any of the stable fractions we have dealt with contain the cobalt as an integral part of a vitamin B_{12}-protein complex remains an open question until we have further purified and assayed these fractions.

D. *The stable cobalto-proteins in other plants.* On finding that cobalt is incorporated in growing *Neurospora* mycelium and that a sizable fraction of this cobalt appears as stable "protein" complexes, we have extended this investigation to a number of other plants. The data are presented in table
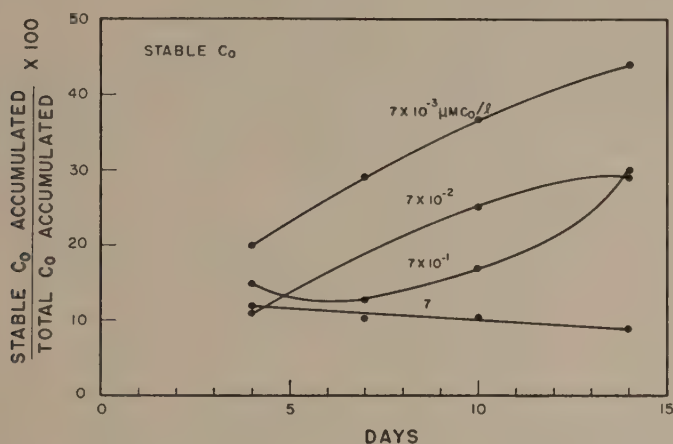
Fig. 7   Stably-bound cobalt as percent of total cobalt accumulated by *Neurospora*. Values for 4 cobalt concentration given above each curve.

3. Tomatoes (Rutgers' Variety) seem to be the most active cobalt accumulators, whereas barley takes up cobalt to a negligible extent. Indeed, in both oats and barley we were working nearly at the limit of sensitivity of our methods, and we can only say that less than 10% of the accumulated cobalt is complexed as stable components. Besides *Neurospora,* the leaves of the musk melon bind a higher percentage of accumulated cobalt as stable "protein" complexes than any other plant we have studied. It may be significant that the protein concentration of their leaves is also very high.

We have found no evidence for any particulate fraction
such as we found in *Neurospora* in any of the green plants.
Preparations of chloroplastids, made according to a modi-
fication of Granick's ('38) method, were free of cobalt (less
than 2×10^{-7} µM/100 mg dry leaf). We may conclude there-
fore that all of the cobalt is present either as ionic or soluble
stable complexes in the cytoplasm.

It should be mentioned that Nickerson and Zerahn ('49)
working with yeast found a very striking accumulation of
cobalt. However, they reported that almost all of this cobalt

TABLE 3

Cobalto-protein content of various plants

*Oats, barley, melon, and tomatoes were grown in water culture in the greenhouse
using the medium of Tsui ('48). Chlorella vulgaris was grown under artificial
illumination at 20°C. with aeration containing 5% CO_2. The medium was that of
Umbreit, Burris and Stauffer ('49)*

PLANT	AGE	Co IN MEDIA, µM/L	Co IN LEAF µM/100 MG	% STABLE Co	MG PROTEIN/ 100 MG DRY WT.
	days				
Barley	21	7×10^{-3}	1.3×10^{-6}	< 10	3.2
Oats	21	7×10^{-3}	4.3×10^{-6}	< 10	2.9
Melon	33	14×10^{-3}	2.5×10^{-5}	24	17.7
Tomato	33	14×10^{-3}	1.7×10^{-4}	13	6.4
Chlorella	18	7×10^{-3}	1.6×10^{-5}	8.3	11.2
Neurospora	11	7×10^{-3}	9.9×10^{-5}	37	29

was soluble in trichloractic acid, in distinction to our stable
complexes. Since they were working at concentrations of
cobalt many thousands of times those we employed, the
phenomena we studied could easily have been masked in
their experiments. The main significance of our results
lies in the fact that the formation of stable "protein" com-
plexes in *Neurospora* is not an isolated instance but occurs
in other plants as well.

F. *Essentiality of cobalt in Neurospora.* The existence of
the metabolic incorporation of cobalt into stable protein com-
plexes would argue either for a cobalt requirement in *Neuro-
spora* or for its replacement of some other element, for in-

stance iron, in such complexes by a "metabolic mistake." These alternatives could easily be resolved by the demonstration that cobalt is required for growth. We have attempted to obtain media deficient in cobalt with respect to growth without success, as has been the case in the past for most animals and the green plants.

Our observation that 7×10^{-4} µM/L of cobalt does not stimulate growth sets an upper limit on the possible requirement for this element, and this is less than one part per billion. Following in detail the procedure of Waring and Werkman ('43) for iron-deficient media, we used not only 8-hydroxyquinoline but also the cobalt reagent, α-nitroso-naphthol, to free our media of heavy metal contaminants. Excellent iron deficiencies were obtained, but there was no evidence for a cobalt requirement.

Neurospora requires .006 ppm iron for 50% maximal growth. As Hutner, Provasoli, Schatz and Haskins ('50) have suggested, this amount of iron might easily be contaminated with sufficient cobalt to supply the full requirement for this element. Indeed, the magnitude of the iron requirement may not be determined by the iron *per se*, but rather by the contaminating cobalt introduced along with it. If this were the case, then addition of cobalt should have a sparing action on iron. We were unsuccessful in demonstrating any such sparing action even when the iron salts were purified of cobalt according to the effective method of Grahame and Seaborg ('38).

If we assume an average molecular weight of 60,000 for cytoplasmic porteins in *Neurospora*, we find that at low cobalt concentrations there is one cobalt atom per 10^3 molecules of protein. Making reasonable assumptions as to cell volume and density, this is within the same order of magnitude of 4.9×10^3 molecules of B_{12} required to produce a single *Euglena* cell as calculated by Hutner et al. ('50). This fact taken in conjunction with our demonstration of stable cobalto-protein complexes is certainly suggestive of physiological function for cobalt in the systems we have studied. However,

a direct demonstration of an essential metabolic function for cobalt must await a precise elucidation of the biochemical reactions directly mediated by its complexes.

SUMMARY

1. Methods utilizing radioactive tracers for studying stably-bound cobalt in the presence of free and ionically bound cobalt are presented.

2. During growth of *Neurospora crassa* ionic cobalt in the medium is accumulated against a concentration gradient. The rate of accumulation only qualitatively follows the growth of the mycelium and protein synthesis. Data for a series of cobalt-ion concentrations are presented.

3. As much as 40% of the cobalt accumulated is present in stable cobalto-protein complexes.

4. The cobalto-proteins can be fractionated into at least two fractions; a soluble fraction comprising 57% of the stably-bound cobalt, and a nearly sub-microscopic particulate fraction.

5. Similar cobalto-proteins have been found in *Chlorella vulgaris* and in the leaves of the musk melon and tomato. Such complexes if present in the leaves of oats and barley are below the sensitivity of the methods used. No cobalt was found in the chloroplastids.

6. Even rigorous cleaning of the media failed to elicit a cobalt deficiency for growth in *Neurospora,* however the amounts of cobalt found in the mycelium are of the same general order of magnitude as the B_{12} concentration required by *Euglena* for growth.

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THE GENERAL FORM OF CIRCULATION IN THE DOGFISH, SQUALUS ACANTHIAS [1]

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FIVE FIGURES

Anatomical studies have clarified the pattern of the structural evolution of the vertebrate circulatory system. The pattern of physiological evolution is less clear. In order to make comparisons, it is necessary to determine the normal character and range of circulatory performance in a variety of lower vertebrates. In the study reported here, an attempt has been made to assess cardio-circulatory physiology in the Elasmobranch, *Squalus acanthias*. Certain circulatory problems are peculiar to fish. The most obvious of these is the question of the influence of the branchial vessels on systemic arterial pressure and blood flow. In fish, the heart pumps unoxygenated blood into an afferent ventral aorta which leads to capillary-like sinusoids in the gills where oxygenation occurs. The effect of this anatomic arrangement on systemic hemodynamics was investigated.

METHODS

Living preparations of dogfish are easy to maintain; but since criteria of normality are not available, and since manipulations easily produce abnormalities, much time was required to develop suitable techniques. Stable, so-called normal, preparations were produced by permitting the fish to respire in cold, well-oxygenated sea water, with its own mechanisms

and not by forced pharyngeal perfusions, and by avoiding external or internal tactile-pressure stimuli which readily induce reflex cardio-inhibition. Anesthesia was unnecessary since the dogfish is remarkably insensitive to pain.

The fish were tied ventral side up in a V-shaped trough immersed in circulating sea water, 11°–17°C. The dorsal aorta was cannulated after cutting off the tail of the fish. A tapered glass cannula provided a leak-proof seal, and occluded the adjacent caudal vein. The ventral aorta was cannulated at its distal bifurcation by a glass cannula or an 18 gauge needle. The cannula was not tied in place, so that complete occlusion of the aorta or any of its branches was avoided. Both operations can be done with almost no bleeding. The arterial pressure was measured with a mercury manometer using sodium citrate as an anticoagulant. Measurements of pressure with a strain-gauge recording through a string galvanometer yielded values in fairly good agreement with those obtained with the mercury manometer.

Pharmacologic studies were made on 150 different fish. In the data reported below, each statement is based upon a study of at least 6 fish. The various drugs were injected subcutaneously near the pectoral girdle. These drugs were explored over a wide range of concentrations, but this exploration was solely for the purpose of establishing a relative dosage which produced maximal effects of a type that seemed significant, and not for the purpose of comparing the activity of a graded series of dosages. The following concentrations were found useful: atropine sulphate, 1 mg; acetylcholine chloride, 1 mg; epinephrine (0.01–0.1 cm^3 of 2.25% racemic solution); histamine, 1 mg; nicotine, 0.1 of a 1% solution; nor-epinephrine bitartrate, 0.018 mg.

RESULTS AND COMMENTS

A. Normal blood pressures and normal circulatory range

Ventral and dorsal aortic blood pressures for unselected so-called normal dogfish are presented in figure 1 a and b.

For 24 fish, the ventral aortic pressures averaged 39/28 mm Hg. For 41 fish, the dorsal aortic pressures averaged 30/23 mm Hg. Frequency distribution plots of the pressures of these fish gave normal unskewed curves (fig. 2 a and b). In the ventral aorta the mode was 38/28 mm Hg. In the dorsal aorta, the diastolic value was usually 24 mm Hg; the systolic values did not peak at a single value but plateaued between 26 and 36 mm Hg. In the ventral aorta, the normal (cf. legend of fig. 1) pressures ranged between 28 to 48 mm Hg during systole, and 20 to 36 mm Hg during diastole. In
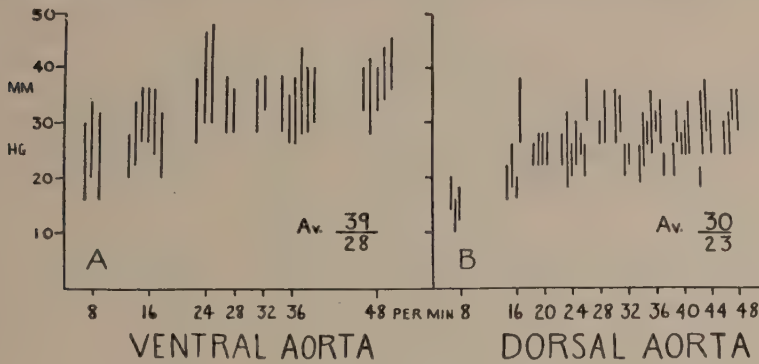
Fig. 1 Heart rates and blood pressures for unselected fish, 36–88 cm in length. Each vertical line represents a different fish. Pressures at heart rate of 8 were not included in the averages.

the dorsal aorta the range was 20 to 38 mm Hg systolic, and 16 to 30 mm Hg diastolic. The pulse pressure was variable, ranging from 6 to 18 mm Hg in the ventral aorta, and from 4 to 14 mm Hg in the dorsal aorta.

The blood pressure varied directly with body length (fig. 3). The data are insufficient for an accurate analysis of this relationship, but the value of both systolic and diastolic pressure increased about 10 to 12 mm Hg as the body length doubled.

The relation of blood pressure to heart rate was more complex. The heart rates encountered at the temperatures

used 11°–17°C.) varied between 16 and 48 per minute, and were frequently in the neighborhood of 36 per minute. Reflex slowing the heart down to 4 were encoutered. After atropine (1 mg), the rate increased to 44–48 beats per minute, which may be considered the intrinsic rate, since there is general
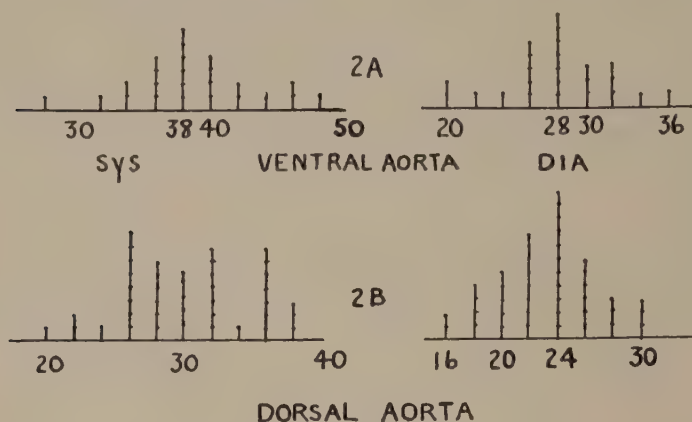
Fig. 2 Frequency distributions of the blood pressures given in figure 1. Systolic pressures on the left, diastolic on the right.
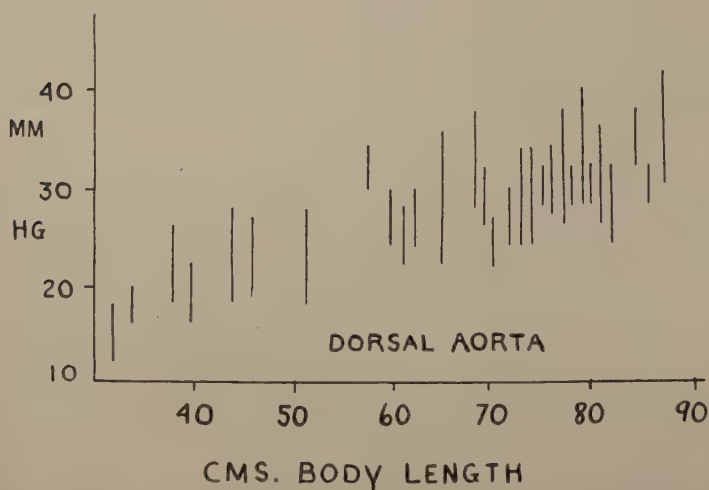
Fig. 3 Relation of dorsal aortic blood pressure to total body length. Each vertical line represents a different fish.

agreement that the vagus alone innervates the Elasmobranch heart. Rates of 36 ± 12 beats per minute may be considered the normal range. Slowing of the heart rate to 16 per minute probably occurs frequently in the dogfish's normal life.

If blood pressures of fish of similar size (72 to 78 cm in total length) are plotted against heart rate (fig. 4), it will be seen that at rates of 24 to 36 beats per minute, the blood pressures (especially the diastolic values) tended to level off.
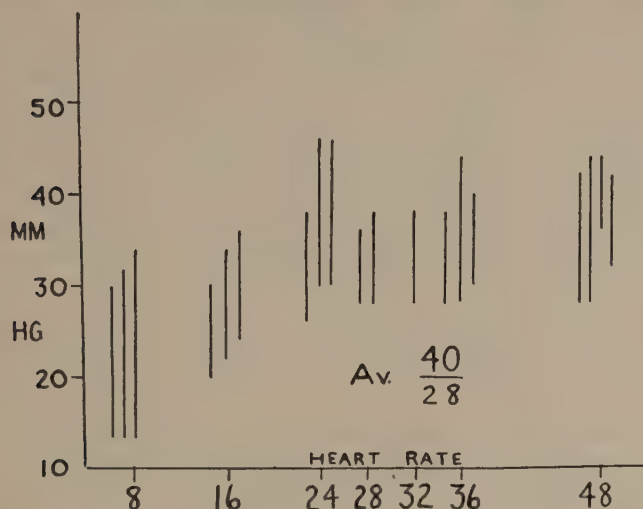
Fig. 4 Relation of ventral aortic blood pressure to heart rate for fish of roughly one size (72–78 cm total length). Pressures at heart rates of 8 were not included in calculations of average pressure.

At about 48 beats per minute, the pressure tended to rise, and below 24 beats per minute, it tended to fall. At minimal rates (4–8 per min.), the ventral and dorsal aortic pressures were characteristically about 32/16 and 16/10 mm Hg, respectively. Individual animals showed a similar behavior of the blood pressure in response to spontaneous variations in heart rate. At any given heart rate, the blood pressure in different animals varied widely. On the other hand, dogfish considered collectively showed a marked tendency to maintain a uniform arterial pressure over a wide range of heart rates.

This result is somewhat at variance with that of Hart ('45) who studied 5 species of fresh water teleosts. He found that blood pressure varied directly in a linear or semi-linear fashion with heart rate, the form of the curve varying with the species.

Pressure in the systemic veins was commonly 0 mm Hg. Negative pressures were not encountered in the veins entering the heart, although with pressor agents such as epinephrine, negative presures of several millimeters mercury developed. The pressure within the pericardial cavity was at atmospheric levels, and not negative as reported by Schoenlein (1895) for *Torpedo*. No evidence of aspiration of blood into the heart was obtained. Indeed, blood was found to circulate when the pericardial and peritoneal cavities and the anterior cardinal sinus were all open to air. The physiological significance, if any, of the huge dilations at the cardiac end of the posterior cardinal veins was not determined.

A satisfactory technique for a detailed study of cardiac output was not developed. Estimations of stroke volume were made by ligating the anterior end of the ventricle during diastole and the posterior end on the initiation of systole and measuring the volume of blood in the heart; by collecting in a small flask the entire output for a few beats; or by measuring the volume displacement per beat in an L-shaped glass cannula which received the entire ventricular output. For dogfish of about 1600 gm, stroke volumes of 0.4 to 1.5 cm³ were encountered "normally," although strokes up to 3 cm³ could be produced by perfusing the ventricle. Distension by this volume, however, induced heart failure. The single most important factor in determining stroke volume appeared to be the amount of blood entering the ventricle. At rapid heart rates stroke volumes were as large as those found at slow rates. Ventricular distension increased both the stroke volume and the heart rate. After atropine the rate increased but the stroke volume decreased. It is evident that the minute volume varies over a considerable range.

Blood volume was estimated by dilution of Evans Blue
(T-1824) in the blood measured spectrophotometrically.
Samples were drawn at 5, 10, and 15 minutes after injection
of the dye into the posterior cardinal vein near the heart.
The resulting curve was extrapolated to three minutes. The
hematocrit was found to be about 30%, and this figure was
used in calculations. The fish studied ranged between 930
and 3600 gm. The blood volume ranged from 71 cm^3 in a
930 gm fish to 250 cm^3 in a 3600 gm fish, and averaged uni-
formly 1 cm^3 of blood for each 15 $\pm$ 2 gm of fish.

The oncotic pressure was low. Plasma from heparinized
blood (pH 7.5–7.6), was dialyzed at 20°C. with cellophane
and celloidin membranes against sea water, pH 8.1 (the
blood is approximately isotonic with sea water), and against
Smith's shark Ringer's solution, pH 7.6. Equilibria was
reached between 2 and 6 cm of water. Meyer ('35) reported
for unspecified Elasmobranchs a range in colloid osmotic
pressure of 3.1 to 6.4 cm of water. Teleost fishes, however,
give much higher values (Keys, '32; Meyer, '35).

B. The effect of the gill-circulation on arterial pressures

The data obtained with unselected fish (fig. 1 a, b) reveal
an average fall in pressure as blood passes through the gills
from the afferent ventral aorta to the efferent dorsal aorta
of 9 mm Hg systolic, and 5 mm Hg diastolic. In 7 dogfish
where pressures were measured in both aortae of the same
fish, the average fall was 8 mm Hg systolic and 2 mm Hg
diastolic. The diastolic fall ranged between 1 and 6 mm Hg.
In test lots of 12 to 16 fish, the limits of this gradient (1 to
6 mm Hg) was not exceeded by the administration of epi-
nephrine, sufficient acetylcholine to slow the heart, epinephrine
or acetylcholine after atropine, or atropine alone.

The diastolic pressure difference is essentially the same
as that obtained by Lutz and Wyman ('32) in studies of
pithed *Squalus*. Similar agreement is found with Lyon ('26)
for *Carcharias*. Calculations from his tracings indicate an

approximate diastolic fall across the gills of 4 mm Hg, and a systolic fall of 12 mm Hg. Since the branchial bed is composed of capillary-like sinusoids, some fall in pressure is to be expected, though this is not necessarily large. Thus, Landis ('34) reports the fall in pressure along the capillaries of frogs as 4 cm H_2O in face of a great variety of initial pressures.
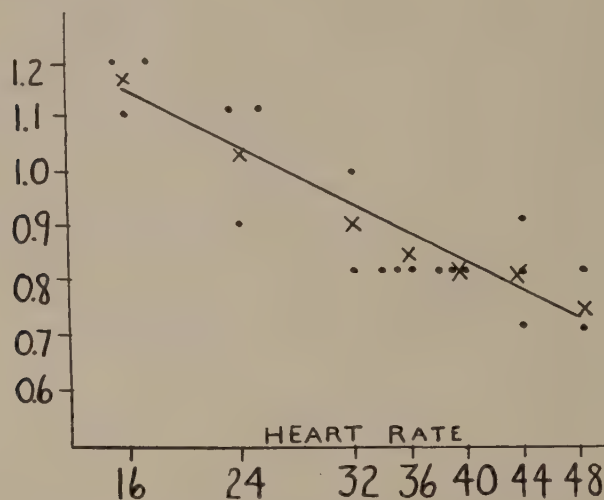
Fig. 5   Effect of atropine on diastolic pressure in the dorsal aorta with different initial (pre-treatment) heart rates. The points recorded are the quotients secured by dividing the post-treated pressure by the pre-treated. Crosses mark the average quotient for each heart rate. The curve was drawn arbitrarily.

Elevation of the arterial pressure by epinphrine resulted in a reduction in the difference between the ventral and dorsal aortic pressures (cf. fig. 5). In a test lot of 16 dogfish, the average pressure differences before epinephrine were 5.6 mm Hg systolic, and 3.5 mm Hg diastolic. After epinephrine, the average differences fell to 1.6 mm Hg systolic, and 1.3 mm Hg diastolic. As found by Wyman and Lutz ('32), the pressure increased more in the dorsal than in the ventral aorta after epinephrine. If blood pressures after epinephrine

are divided by pressures before epinephrine the following
average quotients result: ventral aorta — systolic 1.18, di-
astolic 1.32; dorsal aorta — systolic 1.37, diastolic 1.50. The
rise in all pressures is therefore roughly 18% greater in the
dorsal aorta than in the ventral aorta. Since histological
examination of the branchial vessels of *Squalus* reveal mus-
cular elements it seems probable that branchial vasomotion
occurs with attendant hemodynamic effects, possibly resemb-
ling those observed by Keys and Bateman ('32) in the eel.

Rhythmic respiratory movements seem to have little in-
fluence on circulatory dynamics. Rhythmic gill movements
alter blood pressure 1 to 2 mm Hg or less with the pressure
changes resulting therefrom transmitted to both aortae. The
blood circulates during complete respiratory inhibition.

In the dogfish, therefore, despite the tandem arrangement
of the respiratory and systemic circuits, the systemic arterial
blood is pulsatile with a hydrostatic pressure which exceeds
its oncotic pressure.

C. Circulatory reactivity

Although there is an extensive literature on circulatory
responses in Elasmobranchs (cf. Babkin, '46; Babkin, Bowie
and Nicholls, '33; Huntsman, '31; McKay, '31; Wyman and
Lutz, '32), the range of responsiveness in the normal animal
is not well defined. Cardio-inhibitory reflexes are easily in-
duced by tactile-pressure stimuli on most parts of the body
(Lutz, '30b, c; Lutz and Wyman, '32). The only accelerator
reflex described by Lutz was one resulting from increased
flow of water through the pharynx. Since it is generally
believed that the only motor innervation of the Elasmobranch
heart is a cholinergic vagus (cf. Babkin, '46), an accelerator
humoral agent formed outside the heart has been postulated.
Huntsman ('31) reported that epinephrine accelerated the
isolated heart, although this was not confirmed by Lutz ('30)
or Hiatt ('43). Although an adrenergic substance has been

demonstrated in the chromophil bodies (Lutz and Wyman, '27), nothing is known of circulating levels of endogenous epinephrine. It is generally agreed that epinephrine causes augmented contraction of the isolated heart.

Direct examination of the systemic arteries of Elasmobranchs shows that they are thin-walled, containing muscle fibers (Babkin, Bowie, and Nicholls, '33, this report) and nerves of uncertain origin (Young, '33). The ventral aorta is an elastic artery. Both epinephrine and acetylcholine have been shown to produce constriction of isolated segments of systemic arteries (Babkin, Bowie, and Nicholls, '33) and to induce a pressor response in the intact animal (McKay, '31). Halsey and Minnich ('38) found a decrease in perfusion rate through the celiac arteries of *Squalus* after epinephrine. Acetylcholine did not produce consistent effects.

In the intact *Squalus*, a variety of cardio-inhibitor reflexes were noted. External chemical stimulation with the following agents produce no change in heart rate or blood pressure despite visible discomfiture to the fish: NaOH, NH_4OH, $(NH_4)_2SO_4$, HCl, H_2NO_3, and oil of cloves. All cardio-inhibitor reflexes were blocked by atropine. No new accelerator reflexes were discovered.

Pharmacological studies on the whole animal give information only on the range of circulatory performance and do not clarify the detailed mechanisms involved. A summary of these pharmacologic effects follows (dosage given in Methods):

Atropine. Atropine raised the heart rate to 44–48 beats per minute. The arterial pulse was shallow and the systolic pressure fell. The effect on diastolic pressure is shown in figure 5. If the initial heart rate was slow, the diastolic pressure rose after atropine. If the initial heart rates increased, the diastolic pressure fell progressively after atropine. This drug did not block the response to pressor agents (epinephrine, nor-epinephrine, acetylcholine, and pitressin), but did block the hypotensive action of acetylcholine and nicotine. Following pressor agents, the pulse pressure of the atropinized fish was markedly increased.

Acetylcholine. As noted repeatedly by others, acetylcholine caused cardiac slowing or arrest, an effect blocked by atropine (cf. McKay, '31). Following cardiac inhibition or atropine, the arterial pressure rose above control levels (McKay, '31). The pressor action of acetylcholine is often of the same order of magnitude produced by epinephrine (cf. below), although following the maximum pressor effect of acetylcholine, an additional pressor effect may be produced by epinephrine. When acetylcholine was given in doses sufficient to cause cardiac slowing, the average quotient derived by dividing the diastolic pressure on inhibition by that before inhibition was: 0.56 ventral aorta (7 fish), and 0.58 dorsal aorta (8 fish).

Epinephrine. Epinephrine caused increased blood and pulse pressures, but did not affect heart rate unless given in heavy doses when slowing resulted. The pressure rise was greater in the dorsal than in the ventral aorta; average quotients from post-treatment pressures divided by pre-treatment pressures were: ventral aorta — 1.18 systolic, 1.32 diastolic; dorsal aorta — 1.37 systolic, 1.50 diastolic. In terms of pressure, the maximum changes observed were 20 mm Hg systolic and 16 mm Hg diastolic. Nor-epinephrine produced approximately same effect as epinephrine. If the gut and various portions of the trunk were cut off from the general circulation by constricting the veins and arteries so that only the head was perfused, a pressor effect was still observed. Following epinephrine, the pressure in the posterior systemic veins fell indicating a more rapid emptying of the central venous reservoirs.

Other drugs. Nicotine had a hypotensive effect reversed by atropine. With the onset of muscular tremor and convulsions due to nicotine poisoning, the arterial pressure fell profoundly, circulation ceased although the heart continued to beat vigorously. Histamine had a slight depressor activity only after atropine and dihydroergokryptine and did not affect heart rate. Ephedrine, tetraethylammonium bromide, physostigmine, and dibenamine all in doses up to 100 mg did not influence blood pressure or heart rate.

In general, the range of variation in arterial pressure in the ventral and dorsal aortae that could be produced by the pharmacologic agents employed in this study ranged from 15 to 50 mm Hg systolic and from 10 to 40 mm Hg diastolic. The dogfish, therefore, possesses a considerable potential for circulatory adjustment which appears to involve both cardiac and peripheral vascular activity. To what extent each contributes is difficult to evaluate. Although peripheral

vasoconstriction and vasodilatation clearly occurs, it is impossible to decide on the basis of evidence available at present whether heart and circulation play equally important complementary balancing roles, the first subject primarily to inhibition and the second, to excitation, or whether the heart dominates the cardiovascular bed, determining by its response the peripheral reaction to any stimulus. Of these possibilities, the first seems the more likely, since it has been shown that pressor agents may invoke a vasoconstrictive response independent of cardiac activity. However, under conditions naturally and normally prevailing, it is possible that cardiac activity is dominant and determinant, the residual potentiality of the vascular bed remaining unexploited because of the lack of an effective automonic system. The dogfish lives in a uniform isothermic environment (Bigelow and Schroeder, '48) which requires no antigravitational or thermo-regulatory adjustments other than migration. It lives also in a well-oxygenated environment. There is actually little need, therefore, for a complex system of vascular checks and balances. Further elucidation of this question awaits more exact quantitative exploration of local and generalized hemodynamic adjustment in fish.

SUMMARY

An analysis of the general form of the circulation in so-called normal dogfish, *Squalus acanthias,* has been made in terms of blood pressure and heart rate at rest and under various experimental conditions. Heart rates up to 48 per minute have been observed; the usual rate is about 36 per minute. The ventral aortic blood pressure averaged 39/28 mm Hg with a range of 28 to 48 mm Hg systolic, and 20 to 36 mm Hg diastolic at normal heart rates. Dorsal aortic pressures averaged 30/23 mm Hg with a range of 20 to 38 mm Hg systolic and 16 to 30 mm Hg diastolic. The blood pressure varied directly with body length. It seemed to vary directly with heart rate but showed a marked tendency to level off at heart rates of from 24 to 36 per minute. Venous pressure

near the heart was 0 mm Hg; no aspirating mechanisms were found. The intra-pericardial pressure was at atmospheric pressure.

The average decrement in blood pressure during the passage through the branchial ''capillaries'' from the ventral to dorsal aorta was 9 mm Hg systolic, and 5 mm Hg diastolic. In different fish the diastolic gradient ranged from 1–6 mm Hg. The limits of the transbranchial gradient were not much affected by changes in arterial pressure induced by drugs. Despite the presence of a proximal capillary-sinusoid bed the dorsal aortic pulse pressure ranges from 4 to 14 mm Hg and the dorsal aortic pressure is substantially higher than the oncotic pressure of the blood.

Cardiac output was determined at 0.4 to 1.5 cm^3 blood for 1600 gm normal fish, with a maximum output of 3 cm^3. Blood volume by the Evans Blue dilution method gave 1 cm^3 blood every 15 ± 2 gm of fish. The oncotic pressure was very low, 2–6 cm H$_2$O.

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HEAT PRODUCTION IN CHILOMONAS [1]

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THREE FIGURES

INTRODUCTION

The nature and efficiency of energy transformations have long been a concern of biologists. Primarily two methods are used in studies of these problems. One is the measurement of respiration in conjunction with quantitative analyses to ascertain the amounts of metabolites disappearing and the amounts of products synthesized. The second, which is employed in this study, is the measurement of heat produced by the organisms when a known quantity of substrate is consumed. These methods are supplementary, and for a complete understanding of the energy balance of an organism both should be employed.

Bayne-Jones and Rhees ('29) were among the first to study the heat output of micro-organisms, using *Escherichia* coli and *Staphylococcus* aureus growing in complex media. The complexity of the media, however, made it impossible for them to attempt any thermodynamic calculations.

The heat produced by yeast synthesizing glycogen while oxidizing acetate or glucose was measured by Winzler and Baumberger ('38). From their measurements and from estimates of the free energies and heats of formation of glycogen, acetic acid, etc., they estimated that 2.88% and 12.2% of the available free energy were used for glycogen synthesis when glucose and acetate, respectively, were the substrates being

oxidized. These results were confirmed by Van Niel and Anderson ('41), on the basis of oxygen consumption measurements.

Hutchens, Podolsky and Morales ('48), using carbon balance sheets and oxygen consumption data for *Chilomonas* paramecium grown in mass culture, calculated that about 17% of the free energy made available by the combustion of acetate was used by the organism for the synthesis of carbohydrate, fat, and protein. To do so, the assumption was made, following Baas-Becking and Parks ('27), that the free energy cost of synthesis of fat and protein is the same as that of an amount of anhydrous glucose of equal carbon content. The authors were aware of the crudity of this assumption, and therefore made a separate calculation of the free energy of formation of starch. They thus were able to obtain a more precise figure for the amount of energy devoted to starch synthesis during normal growth. The thermodynamic data which would permit a similar calculation for protein are not known. It is possible, however, to make a fairly good estimate of the amount of energy devoted to fat synthesis, and thus to add to the energy balance sheet for *Chilomonas* begun by Hutchens et al.

The present study of heat produced by chilomonads under various conditions was undertaken to obtain answers to the following questions:

1. What fraction of the heat theoretically available from the complete oxidation of substrates is actually produced?

2. Is the heat produced (as per cent of that theoretically available) the same with and without a nitrogen source, i.e., when the products of synthesis are varied?

3. Is the amount of heat produced in each case consistent with the free energy and entropy values assumed by Hutchens et al. ('48)?

4. Is any of the free energy available from the conversion of ethyl alcohol to acetate captured by *Chilomonas?*

5. What fraction of the available energy is devoted to the synthesis of fat?

MATERIALS AND METHODS

A. Organisms. The chilomonads used in these experiments
were grown in pure culture at 25°C. in the culture solution
described by Hutchens ('48). The original pH value of all cul-
tures was 6.0. Samples were taken for counting at the begin-
ning and again at the end of each experiment in which heat
production was measured. The counting technique has also
been described by Hutchens ('48). In experiments in which
the organisms were given substrate in the presence of am-
monium salts, the solution in which they were washed and
suspended was identical with the culture solution except that
an equimolar amount of NaCl replaced the sodium acetate.
In experiments in which the assimilation of added substrate
took place in the absence of ammonium salts, an equivalent
amount of Na_2SO_4 was added to replace the NH_4Cl and
$(NH_4)_2SO_4$. The carbon sources used were sodium acetate
and ethyl alcohol.

B. Apparatus. A differential calorimeter system was used.
Similar apparatus has been described by Swietoslawski ('46)
and White ('10, '14). Two highly evacuated, 300 cm³ capacity,
balloon shaped, Dewar flasks matched as nearly as possible in
their characteristics were used. The culture was put into one,
and an equal volume of wash solution into the other (null
flask). A Beckmann thermometer was employed to give the
temperature of the null flask. The temperature difference be-
tween the null flask and the reaction flask was measured by a
24 junction thermopile, the reference junctions of which were
put in the null flask. The flasks were almost totally immersed
in a water bath, which was kept constant within at least
± .002°C., as measured by a second Beckmann thermometer.
A more detailed description of the apparatus follows.

A 24 junction thermopile was constructed (White, '10). The ele-
ments chosen were cupron (38 gauge) and manganin (33 gauge),
and gave an Emf. of .985 $\frac{mv}{°C.}$ as found by repeated calibration with
two Beckmann thermometers. The internal resistance of the thermo-
pile was calculated to be about 800 ohms from the known specific
resistances and lengths of the wires used, and was actually measured

to be 820 ohms. The total length of the thermopile in each flask was 6 inches, with 5 inches exposed between the flasks. The junctions were vertically staggered in a tapered glass tube, filled with mineral oil and sealed with paraffin. This served to prevent the accidental entrance of water, and to prevent the transmission of strain to the junctions. The two cupron wires from which the potential was to be measured were soldered to copper plates, ¾ inches square. The copper plates were separated by mica and the whole assembly was pressed together by a clamp and immersed in a small oil bath. This in turn was nearly submerged in the constant temperature bath. This minimized the possibility of having stray thermal Emf's arise as a result of variations in temperature of the junctions between the thermopile and the measuring apparatus. The entire system, including both flasks, the oil bath, and the exposed parts of the thermopile was covered by two copper cans appropriately perforated to admit the Beckmann thermometer and other instruments. Both cans dipped into the water bath, thus helping maintain the air above the flasks at bath temperature. Room temperature was kept at $25.2 \pm .5°C$.

The leads from the thermopile were conducted via a shielded cable into a switching box, containing switches, I, II, III, IV, and V (fig. 1). The switches were all double-pole, double-throw, pure copper, Leeds and Northrup switches, especially designed to be free of contact potentials. The box was sealed with felt and the switches were operated by wooden dowel rods 12 inches long. In this box were located all the resistors, both fixed and variable. The shafts of the variable resistors were surrounded by cotton at their points of exit from the box, and were capped with plastic knobs by which they were adjusted. All wires outside the box (i.e. between the box, the type K potentiometer, and the galvanometer), were shielded, the shield being connected to ground.

The galvanometer used was a Leeds and Northrup type HS reflecting galvanometer, with the following characteristics: Sensitivity .1 mv/mm, C.D.R. 26 ohms, period 7.4 sec., and an internal resistance of 18.4 ohms.

All measurements were begun with a standard set of switch positions, which were: Switch I in the circuit position (C in fig. 1), switch II in the parasitic position (P in fig. 1), and switches III and IV in the neutral position. In this standard position the batteries B and the thermopile T were disconnected, and the thermopile was replaced by an equivalent resistor (Z in fig. 1). The scale on which the galvanometer was read was then adjusted to zero deflection, thus eliminating all parasitic Emf's. The measurement of the temperature difference between the flasks was then made in two steps.

Step 1. With switch I in the circuit position, switch II was put in the "read" position (R in fig. 1), switches III and IV were closed, and the variable resistors adjusted until a zero deflection was obtained. The direction in which switch IV was closed depended on which junction of the thermopile was hotter.

Step 2. The above balancing operation resulted in the establishment of an Emf across r_2 exactly 1000 times as large as that across
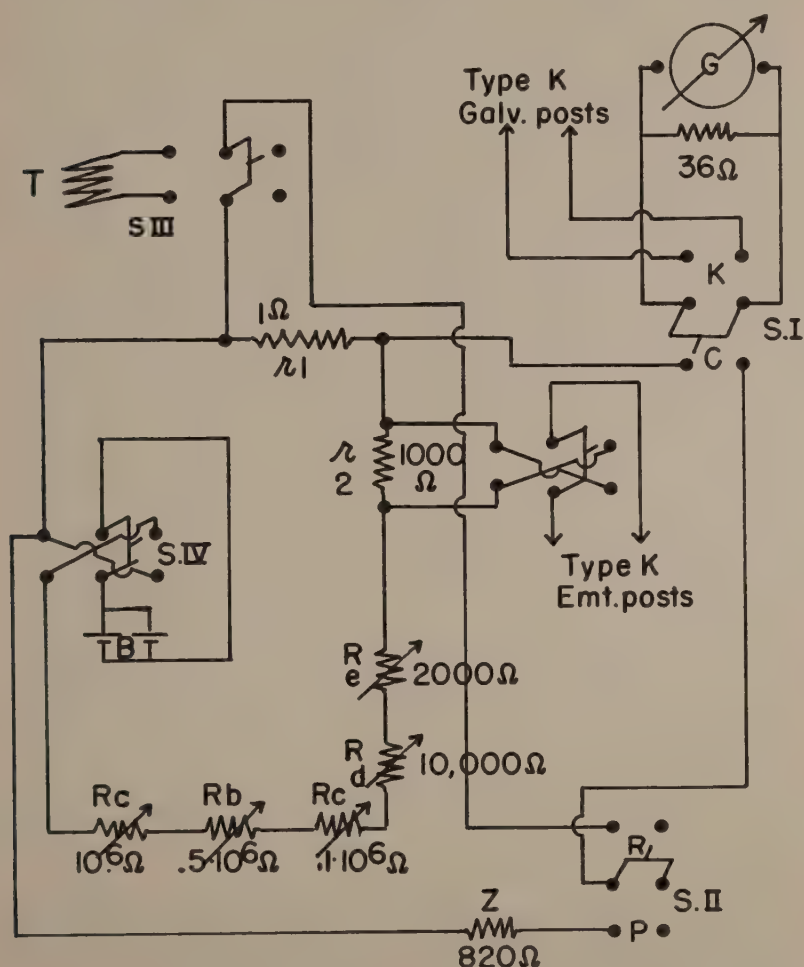
Fig. 1 Circuit diagram of apparatus used. The symbols are explained in the text.

r_1. This was the Emf finally measured by the potentiometer, a Type K_2 Leeds and Northrup instrument. Step 2 thus consisted of changing switch I from the circuit position (C) to the Type K position (K). The Emf across r_2 was led to the potentiometer through switch V. This switch was used to insure an Emf of the proper sign with respect to the potentiometer circuit. The potentiometer thus read 1000 times the voltage which the thermopile developed at the time of reading, i.e. the time at which switch I was thrown from C to K.

The cooling curves of the flasks were measured and the cooling (heating) constants calculated. In degrees lost (gained) per minute per °C. difference between flask temperature and bath temperature, the constants had an average value of 3.5×10^{-4} when each contained 200 cm³ of water. Since the temperature increment during any experiment was less than .05°C., correction for heat gain or loss was unnecessary.

The heat capacity of each flask was calculated from the passage of a known current for a known time through a 98.0 ohm resistor. This was repeated several times with different amounts of water in the flask. Each flask acted as if it had a heat capacity equivalent to 18 cm³ of H_2O, with the probable limits of error being ± 1 cm³. Thus, with 200 ml of H_2O in each flask, the heat capacities were known within ± 0.5%.

A gas delivery system was set up to supply the organisms with oxygen. For this purpose, three sintered-glass disk saturators were used in series. The first two contained distilled water. The third contained a sodium chloride solution of the same ionic strength as the culture solution. The second and third saturators were both immersed in the bath, while the first was suspended above the bath. From the third saturator, the gas went past a constant pressure head of 15 inches of water, thence via a Y tube to a pair of matched flow control capillaries, and finally into glass delivery tubes, one for each flask. The pressure drop across the capillaries (which were submerged in the bath), theoretically leads to less than 100% saturation of the gas led into the flask. However, this unsaturation is small (ca. 0.5%) and heat loss through evaporation of water is corrected for by leading the same amount of

gas of the same per cent saturation into each flask. Repeated observations showed that the rate at which the gas was supplied during the experiment was sufficiently low to eliminate any need for correction for cooling caused by the gas. The solution in each flask was saturated with oxygen at the beginning of each experiment. When the substrate was introduced, oxygen was bubbled into each flask for a period of about two minutes. In addition to the above periods of bubbling, the gas was also supplied for 15 seconds after each measurement.

C. Experimental procedure. The chilomonads used were taken from mass cultures having a population of $1.5-2 \times 10^5$ cells/ml. The organisms were either in the logarithmic growth phase or had just begun the phase of decreasing growth acceleration. Two hundred and fifty milliliters of cell suspension were centrifuged for 5 minutes at $100 \times$ gravity. The supernatant solution was decanted, and the organisms were resuspended in the appropriate wash solution. The organisms were then sedimented at the same acceleration for another 5 minutes, and this wash solution was decanted. The organisms were resuspended in wash solution and were now assumed to be free of exogenous substrates. Two hundred milliliters of this cell suspension were then put into the reaction flask, and a sample taken for counting. The system was covered, and readings were taken at 15-minute intervals until a steady state of heat production was attained (about 100 minutes).

When the steady state was attained, an opening in the copper cans above the reaction flask was unplugged, and a 2 cm^3 pipette containing the substrate was inserted into the flask and allowed to drain. The system remained open for an interval of about one minute, after which it was closed and a temperature measurement was taken. Gas was bubbled through the flask during the addition of the substrate to oxygenate and mix the substrate through the solution. If the addition of the substrate caused a change in temperature of more than $.001°C.$, this amount was added to or subtracted from all subsequent readings, in order to make the temperature versus time curves continuous at the time of addition of the substrate. Since the

induction period of the organisms under the conditions of the
experiment was always 5 minutes or more, no error was intro-
duced by this procedure.

Readings were taken at approximately 15-minute intervals
until extra heat production had ceased. The system was then
opened, and a sample taken for a final cell count. A curve of
temperature rise versus time was plotted, and the net tem-
perature change was obtained by extrapolating the curve, as is
shown in figure 2.
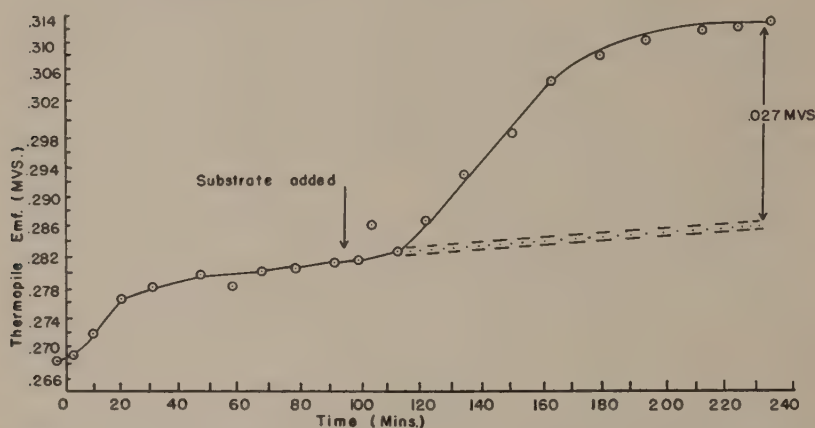
Fig. 2   Typical variation of temperature versus time. Temperature expressed
in mv. Substrate is 61 × 10⁻⁶ moles of sodium acetate.

The difficulties typically encountered in using this method
of extrapolation to obtain the net temperature change are well
illustrated by this curve. The dotted line is a least squares
line, calculated from the first 4 points just preceding the addi-
tion of substrate. The dashed lines have the same slope as the
least square lines, but their intercepts are two standard errors
above and below the least squares line. It will be seen that the
extrapolated value for the temperature can be in error by
about 0.001°C. in .027°, or about ± 4%. This is the approxi-
mate error involved in most of the experiments. In none of
the experiments reported was the error involved in the extra-
polation larger than 6%.

METHODS OF CALCULATION AND EXPERIMENTAL RESULTS

When acetate is utilized as a substrate by *Chilomonas*, the chief products of synthesis are starch, protein, and fat. The data of Hutchens et al. ('48) show that 38.8% of the cell carbon is in starch, 42.0% in protein, and 19.2% in fat. In the absence of NH_4^+ ions as a source of nitrogen, no protein is synthesized. Probably no great error results from assuming that, in the absence of NH_4^+, the carbon which would have been used for protein synthesis is incorporated into starch, and that the amount of fat synthesized remains unchanged. Thus we shall assume that in the absence of NH_4^+ ions 81% of assimilated carbon goes into starch and 19% into fat. We shall also calculate the per cent efficiencies both in the presence and absence of a nitrogen source as if the only product of synthesis were starch, so that we may compare these values to older values in the literature which were calculated using similar assumptions.

A. *Heat available from acetate oxidation*

The following equations may be written, using values given in table 3.

$$CH_3COONa + 2O_2 + NaH_2PO_4 \longrightarrow Na_2HPO_4 + 2CO_2 + 2H_2O$$
$$\Delta H_{298} = -205,720 \text{ cals.} \tag{1}$$
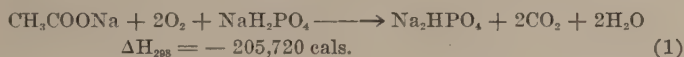
This is the equation we shall use for subsequent calculations involving heat produced by oxidation of acetate. If to this equation we add

$$Na_2HPO_4 + CH_3COOH \longrightarrow CH_3COONa + NaH_2PO_4$$
$$\Delta H_{298} = -880 \text{ cals.} \tag{2}$$

where all substances are in aqueous solution, we then obtain as a value for the heat of combustion of acetic acid in dilute solution

$$CH_3COOH \text{ (aq.)} + 2O_2 \text{ (gas)} \longrightarrow 2H_2O + 2CO_2 \text{ (gas)}$$
$$\Delta H_{298} = -206,600 \text{ cals.} \tag{3}$$
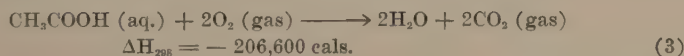
which is in good agreement with the value used by Winzler and Baumberger ('38) for the heat of combustion of acetic acid.

B. The heat cost of synthesizing starch from acetate

From data tabulated in table 3 we may write:

$$C_6H_{10}O_5 \text{ (solid)} + 6O_2 \text{ (gas)} \longrightarrow 6CO_2 \text{ (gas)} + 5H_2O$$
$$\Delta H_{298} = -677{,}550 \text{ cals.} \tag{4}$$
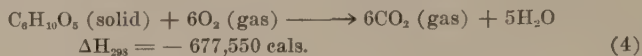
Since in our experiments starch was presumably present fully hydrated, we must add to this the heat of hydration of anhydrous starch. Rodewald ('00) has measured the heats of hydration of several starches, and obtained values ranging from 28–32 cals./gm when an excess of water was added to dry starch at 0°C. He observed that most of the heat change occurred during addition of the first three moles of water. It is unlikely that any significant amount of starch dissolved under these circumstances. We shall take the average figure, 30 cals./gm (equal to -4860 cals./$C_6H_{10}O_5$ unit) as the heat of hydration of starch at 0°C. In the absence of data at other temperatures, we are forced to assume that this value holds at 25°C. We neglect the heat of solution of the hydrated starch which is probably negligible. With these reservations we write:

$$C_6H_{10}O_5 \text{ (solid)} + 3H_2O \longrightarrow C_6H_{10}O_5 \cdot 3H_2O \ \Delta H_{298} = -4860 \text{ cals.} \tag{5}$$
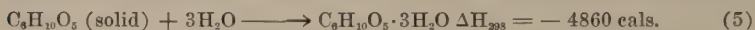
Subtracting equation (5) from equation (4), we have

$$C_6H_{10}O_5 \cdot 3H_2O + 6O_2 \longrightarrow 6CO_2 + 8H_2O \ \Delta H_{298} = -672{,}690 \text{ cals.} \tag{6}$$

Subtracting from (6) the heats of formation of CO_2 and H_2O, we then obtain

$$6C + 8H_2 + 4O_2 \longrightarrow C_6H_{10}O_5 \cdot 3H_2O \ \Delta H°_{298} = +438{,}050 \text{ cals.} \tag{7}$$
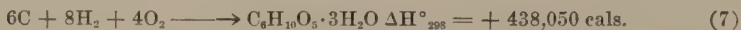
as the heat of formation of the hydrated hexose unit. If we subtract from this the heat of formation of three water molecules and the heat of hydration of the hexose unit, we obtain $-228{,}230$ cals., which is close to the figure used by Winzler and Baumberger ($-226{,}590$ cals.) as the heat of formation of the anhydrous hexose unit of glycogen. We may therefore write

$$CH_3COONa + NaH_2PO_4 + 2/3\ H_2O \longrightarrow 1/3\ (C_6H_{10}O_5 \cdot 3H_2O) + Na_2HPO_4$$
$$\Delta H_{298} = +18{,}510 \text{ cals.} \tag{8}$$

This may be compared with the equation from Hutchens et al. ('48)

$$CH_3COO^- + H^+ + \frac{1}{72} H_2O \longrightarrow 1/3 \text{ Amylose hexose unit } \Delta F°_{298} = + 18,300 \text{ cals.}$$

(9)
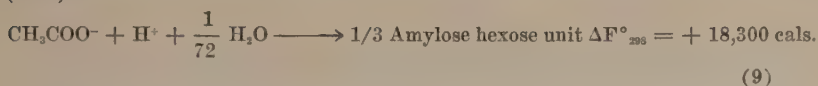
The values indicate that the entropy change involved in formation of starch from acetate is small.

C. The heat cost of synthesizing fat from acetate

The measurements of Hutchens et al. ('48) indicate that 19% of the acetate used for synthesis is used for the formation of fat. This finding was confirmed in the course of the present study. In addition, preliminary attempts to characterize the fats have been made. Fats were extracted from *Chilomonas* with boiling 1 : 3 ether-ethanol, the solvent evaporated, and the fats taken up in petroleum ether. Analysis of the fats for total fatty acid and cholesterol were made following Bloor ('28). The results indicated that only 2.5–3.0% of the total lipids were cholesterol-like material, as indicated by the color developed in 15 minutes with acetic anhydride and H_2SO_4 in chloroform. The fatty acids required 3.5 ml of .1N $K_2Cr_2O_7$ per milligram on wet combustion in sulfuric acid which is near the values usually found for mixed fats from animal sources. The presence of glycerol in hydrolysates extracted for cholesterol and fatty acids was demonstrated by the acrolein test. Since an insignificant fraction of the fats as extracted dissolved in cold 10^{-3}N KOH it seems that for the present purposes we may assume that the fats closely resemble such compounds as triolein, tripalmitin, and tristearin.

We write therefore for triolein,

$$28\tfrac{1}{2}CH_3COONa + 28\tfrac{1}{2}NaH_2PO_4 \longrightarrow \text{Triolein} + 28\tfrac{1}{2}Na_2HPO_4 + 23O_2 + 5H_2O$$
$$\Delta H_{298} = + 2,545,670 \text{ cals.} \tag{10}$$
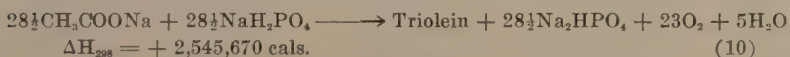
This amounts to + 89,321 cals. per mole of acetate converted to fat. Similar calculations give + 90,546 cals. for tripalmitin, and + 92,000 cals. for tristearin. We shall adopt the average

value of 90,600 cals. for the conversion of a mole of acetate to fat.

D. Acetate assimilation in the presence of ammonia

The foregoing equations provide a basis for calculations of the amounts of acetate oxidized and assimilated. Thus using equations (1) and (8) and assuming that starch only is synthesized we write

$$nCH_3COONa + 2nO_2 + nNaH_2PO_4 \longrightarrow nNa_2HPO_4 + 2nCO_2 + 2nH_2O$$
$$\Delta H_{298} = - \text{ n } 205{,}720 \text{ cals.} \tag{1a}$$

$$mCH_3COONa + mNaH_2PO_4 + \frac{2m}{3}H_2O \longrightarrow \frac{m}{3}(C_6H_{10}O_5 \cdot 3H_2O) + mNa_2HPO_4$$
$$\Delta H_{298} = + \text{ m } 18{,}510 \text{ cals.} \tag{8a}$$
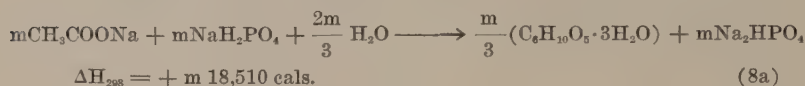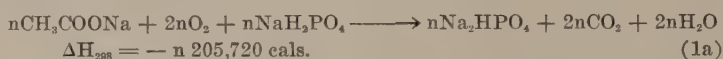
Assigning various values to n and m such that $n + m = 1$ and adding the above equations we obtain values for the expected heat per mole of added substrate for various per cents oxidized and assimilated. The results of such a calculation are presented graphically in figure 3. A similar set of equations taking into account the fact that 0.19 m moles of acetate are converted to fat and 0.81 m moles of acetate are converted to starch can be written. The expected heat production with both starch and fat being synthesized can thus be obtained. The results of such a calculation are also presented in figure 3.

Table 1 gives results of experiments in which extra heat production was measured following addition of various amounts of acetate to suspensions of chilomonads. Columns 4 and 5 of this table give the values derived from figure 3 for the per cent of acetate oxidized.

It will be seen from table 1 that, in the presence of NH_4^+ ions, assuming only starch to be synthesized leads to the result that $54.1 \pm 2.3\%$ (2 S.E.) of the added acetate was oxidized. Taking fat synthesis into account leads to the conclusion that $56.7 \pm 2.4\%$ (2 S.E.) was oxidized. This increase in the percentage apparently oxidized results from the greater heat cost of synthesis of fat as compared to starch.
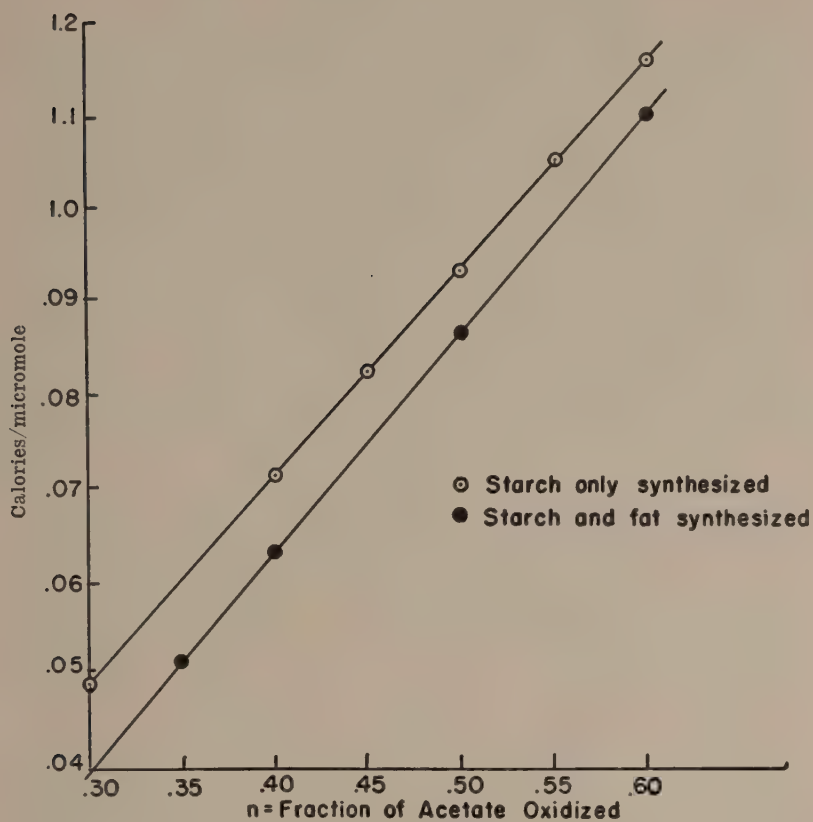
Fig. 3 Graph of heat production expected per micromole of acetate oxidized.

TABLE 1

Heat production induced in Chilomonas by the addition of acetate in the presence of a nitrogen source

ACETATE ADDED	OBSERVED HEAT PRODUCTION	CALORIES/ μ MOLE	% ACETATE OXIDIZED	
			Starch only synthesized	Starch and fat synthesized
μ moles	calories			
61.0	6.14	.1006	53.2	55.5
61.0	5.86	.0961	51.2	54.0
61.0	6.08	.0997	52.7	55.1
71.76	7.88	.110	57.2	60.0
35.88	3.89	.108	56.4	59.2
	Average		54.1%	56.7%

E. Acetate assimilation in the absence of ammonia

The above calculations lack precision because no account could be taken of the heat cost of protein synthesis. By eliminating the nitrogen source from the medium protein synthesis can be prevented. Starch and fat should then represent the bulk of materials synthesized. In table 2 are presented the results of experiments in which extra heat production was measured following addition of acetate to suspensions of chilomonads in a nitrogen free medium.

Columns 4 and 5 of table 2 give the values derived from figure 3 for the per cents of added acetate oxidized. Assuming

TABLE 2

Heat production induced in Chilomonas by the addition of acetate in the absence of a nitrogen source

ACETATE ADDED	OBSERVED HEAT PRODUCTION	CALORIES/ μ MOLE	% ACETATE OXIDIZED	
			Starch only synthesized	Starch and fat synthesized
μ moles	*calories*			
48.16	3.15	.0654	37.7	41.3
48.16	3.15	.0654	37.7	41.3
98.32	6.33	.0644	37.2	40.9
48.16	2.72	.0565	33.8	37.5
	Average		36.6%	40.2%

that 19% of the assimilated acetate was converted to fat leads to the result that $40.2 \pm 1.9\%$ (2 S.E.) was oxidized. Because the heat cost of the major products of synthesis was taken into account these figures have, we feel, considerable reliability. The per cent of acetate assimilated is higher than that observed for yeast by Winzler and Baumberger ('38) under similar conditions.

F. The heat of formation of starch and fat from ethanol

When starch is synthesized from ethanol, acetate is presumably an intermediate. Adding the values of heat of combustion

and of solution of alcohol obtained from table 3, and subtracting equation (3) from the result, we get

$$C_2H_5OH \text{ (aq.dil.)} + O_2 \text{ (gas)} \longrightarrow CH_3COOH \text{ (aq.dil.)} + H_2O$$
$$\Delta H_{298} = -119{,}696 \text{ cals.} \tag{11}$$
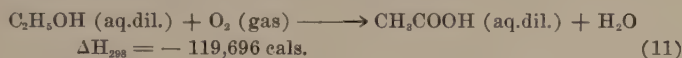
This enables us to write equations analogous to (1a) and (8a).

$$nC_2H_5OH + 3nO_2 \longrightarrow 2nCO_2 + 3nH_2O \ \Delta H_{298} = -325{,}060 \text{ cals.} \tag{12}$$

$$mC_2H_5OH + mO_2 \longrightarrow mCH_3COOH + mH_2O \ \Delta H_{298} = -m119{,}696 \text{ cals.} \tag{11a}$$

$$mCH_3COOH + \frac{2m}{3} H_2O \longrightarrow \frac{m}{3} (C_6H_{10}O_5 \cdot 3H_2O) \ \Delta H_{298} = +m17{,}630 \text{ cals.} \tag{13}$$
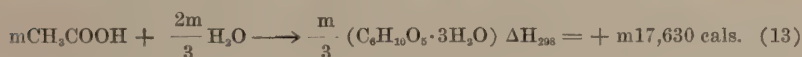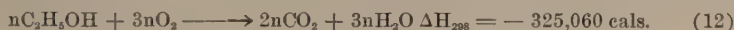
Equations (11a) and (13) imply that all the alcohol could be converted into starch with an accompanying evolution of heat amounting to 102,066 cals./mole.

TABLE 3

Heat of formation, combusion, and solution of various substances (in calories)

SUBSTANCE	STATE	ΔH_{298} FORMATION	ΔH_{298} SOLUTION	ΔH_{298} OF FORMATION OF AQUEOUS SOL.	ΔH COMBUSTION
CH_3COONa	dilute aqueous			−175,100	
Na_2HPO_4	dilute aqueous			−420,620	
NaH_2PO_4	dilute aqueous			−364,500	
H_2O	liquid	−68,320			
CO_2	gas	−94,030			
O_2	gas	0			
$C_6H_{10}O_5$	solid				−677,550
CH_3COOH	liquid	−117,600	−500	−118,100	−206,600
C_2H_5OH	liquid	−66,330	−2540	−68,870	−327,600
$C_6H_{10}O_5 \cdot 3H_2O$	hydrated solid	−438,050			
Triolein	liquid	−503,660			
Tripalmitin	solid	−588,410			
Tristearin	solid	−632,270			

These values and the values found in table 5 are taken from the International Critical Tables, Volume V, and from a tabulation of such values kindly supplied by Professor Borsook.

To obtain the heat of formation of a fat unit from ethanol, we add to equation (11) the average value for the heat of formation of a fat unit from acetic acid, which can be calculated from equations (10) and (2). The average value of the heat of formation of a fat unit from ethanol is then — 29,976 cals./ mole of ethanol.

G. *The fraction of ethanol assimilated in the absence of ammonia*

Data from experiments conducted using ethanol as a carbon source and energy source for *Chilomonas* are presented in

TABLE 4

Heat production induced in Chilomonas by the addition of ethyl alcohol in the absence of a nitrogen source

ALCOHOL ADDED	OBSERVED HEAT PRODUCTION	CALORIES/ μ MOLE	% ALCOHOL OXIDIZED	
			Starch only synthesized	Starch and fat synthesized
μ moles	*calories*			
60.18	7.84	.130	12.8	17.5
30.09	4.06	.135	15.0	19.6
60.18	7.70	.128	12.0	16.5
30.09	3.82	.127	11.5	16.2
		Average	12.8%	17.5%

table 4. Using equation (11a), (12) and (13), a graph analogous to figure 3 was constructed. Assuming only starch to be synthesized the results indicated on the average 13% of the ethanol was oxidized and 87% assimilated in the absence of ammonia.[3] If one takes into account the heat changes accompanying fat synthesis these same results indicate oxidation of 18% of the added ethanol (table 4).

[3] In other experiments (unpublished), we have found that the average net R. Q. for *Chilomonas* oxidizing ethanol in the absence of a nitrogen source is about .25. This corresponds to the value expected if all the ethanol were oxidized to acetate and 16% of the acetate were oxidized to CO_2 and H_2O. It is seen that the respiratory data are not incompatible with the calorimetric data.

In a single experiment in which ammonia was present the results indicated complete oxidation of 16% of the ethanol with the assimilation of 84%. This is in the same range as the values obtained in the absence of NH_4^+, and indicates that no appreciable change was made by the inclusion of a nitrogen source. More experiments are required to establish this point.

TABLE 5

Free energies of formation and concentration of various substances

SUBSTANCE	STANDARD STATE	ΔF°_{298} CALS.	CONCENTRATION USED IN EXPERIMENTS
CH_3COO^-	aq a = 1 molal	—90,130	1.4×10^{-5} M average
CH_3COOH	aq a = 1 molal	—96,620	1×10^{-5} M average
C_2H_5OH	aq a = 1 molal	—43,380	1×10^{-4} M average
H_2O	pure liquid	—56,690	pure liquid
Na^+	aq a = 1 molal	—62,588	2×10^{-2} M average
CO_2	p = 1 atmos.	—94,240	0.0003 atmos.
Triolein	liquid	—104,860	....
Tripalmitin	solid	—170,860	...
Tristearin	solid	—169,180	....
HPO_4^-	aq a = 1 molal	—267,100	8.07×10^{-3} M
$H_2PO_4^-$	aq a = 1 molal	—257,270	5.3×10^{-4} M
O_2	p = 1 atmos.	0	.2 atmos.
H^+	aq a = 1 molal	0	10^{-6}
Starch per hydrated hexose unit	sat. soln.	—217,851	sat. soln.

These values and the values found in table 3 are taken from the International Critical Tables, Volume V, and from a tabulation of such values kindly supplied by Professor Borsook.

FREE ENERGY CHANGES

A. Free energy available from combustion of acetate

Hutchens et al. ('48) presented the following equation:

$$CH_3COO^- + H^+ + 2O_2 \longrightarrow 2CO_2 + 2H_2O \quad \Delta F^\circ_{298} = -211,733 \text{ cals.}$$
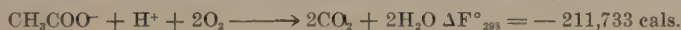
Correcting for our experimental conditions (table 5), we obtained values of ΔF_{298} of $-206,379$ cals. at the beginning of the experiment. The values increase to $-205,014$ cals. and $-204,649$ cals. by the time 90% and 99% of the acetate have disappeared. We shall adopt a mean value of $-206,000$ calories.

B. Free energy cost of starch synthesis

Hutchens et al. ('48) give for the free energy of the reaction

$$3CH_3COO^- + 3H^+ + \frac{1}{24} H_2O \longrightarrow \text{amylose hexose unit}$$

a value of $\Delta F°_{298} = + 54,901$ cals.
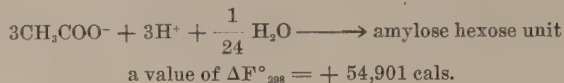
Again correcting this for our experimental conditions, we obtain an average of ΔF_{298} of $+ 95,274$ cals., or $+ 31,758$ cals. per acetate converted to starch.

C. Free energy cost of synthesis of fat from acetate

Assuming as in previous calculations that the major part of the fat synthesized consists of neutral fats containing fatty acids such as palmitic, stearic, or oleic acids, we write for the reaction involving synthesis of triolein:

$$28.5CH_3COO^- + 28.5H^+ \longrightarrow \text{Triolein} + 5H_2O + 23O_2 \quad \Delta F°_{298} = + 2,180,395 \text{ cals.}$$

Correcting this for our experimental conditions, we obtain $\Delta F_{298} = + 2,580,704$ cals. or $+ 90,547$ cals. per acetate converted to triolein.

Similar calculations for tristearin and tripalmitin show $\Delta F_{289} = + 94,210$ cals. and $+ 93,000$ cals. respectively. We shall adopt an average value of 92,600 cals.

D. Free energy devoted to synthesis

The foregoing calculations permit us to make an estimate of the fraction of the free energy made available from oxidation of acetate which is accounted for by synthesis achieved. In those experiments in which no nitrogen source was present and therefore no protein was synthesized, we can calculate this with considerable accuracy. Thus, if we assume only starch to be synthesized, 36.6% of the acetate is oxidized and 63.4% assimilated (table 2). The per cent of available free energy required is

$$\frac{.634 \times 31,758 \times 100}{.366 \times 206,000} = 26.7\% \cdot$$

If, more realistically, we assume both starch and fat to be synthesized with 40.2% of the acetate being oxidized (table 2), 48.4% being synthesized to starch and 11.4% being converted to fat, the per cent of available free energy required is

$$\frac{(.484 \times 31.758 + .114 \times 92,600) \times 100}{.402 \times 206,000} = 31.3\%$$

When a nitrogen source is present and the products of synthesis include protein as well as starch and fat, calculations having any real meaning are impossible. The per cent of available acetate assimilated as calculated from thermal data is in doubt because of lack of knowledge of the heat cost of protein synthesis. The free energy cost of protein synthesis is unknown. Simply for purposes of comparison we present a calculation based on the assumption that the free energy cost of synthesis of protein equals that of synthesizing an amount of starch of equal carbon content. Taking the value from table 1 for synthesis of starch only, we have

$$\frac{.459 \times 31,758 \times 100}{.541 \times 206,000} = 13.1\%$$

as the per cent of available free energy devoted to synthesis. Taking fat synthesis into account would change this figure to 17.8%.

It thus appears that in the presence of a nitrogen source less of the available free energy is captured in synthetic processes. This may be true. The possibility remains, however, that protein synthesis costs more from the free energy standpoint than starch synthesis and that the assumption made above is unjustified. The important point to be made is that the nature of the end products strongly influences the apparent "free energy efficiency of growth." It should also be pointed out that the energy capture by *Chilomonas* in the absence of a nitrogen source is remarkably high. Under almost identical conditions Winzler and Baumberger ('38) found yeast to capture only 12.2% of the energy available from acetate oxidation.

E. Free energy available from ethanol oxidation

In subsequent calculations we shall assume that all ethanol added to the cultures was oxidized at least to acetic acid. This is consistent with the observation that such cultures quickly become acid. It is also consistent with the commonly accepted metabolic schemes. We therefore write:

$$C_2H_5OH + O_2 \longrightarrow CH_3COO^- + H^+ + H_2O \quad \Delta F°_{298} = -103,440 \text{ cals.}$$

Under our experimental conditions (table 5)

$$\Delta F_{298} = -110,676 \text{ cals. on the average.}$$

A fraction of the ethanol added is completely oxidized. For the reaction

$$C_2H_5OH + 3O_2 \longrightarrow 2CO_2 + 3H_2O \quad \Delta F°_{298} = -315,170 \text{ cals.}$$
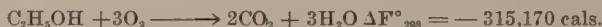
Under our conditions $\Delta F_{298} = -316,465$ cals. on the average.

F. The free energy cost of synthesis of starch and fat from ethanol

We come now to the question of the work done in synthesis of starch and fat from ethanol. The critical question in this connection is the pathway of synthesis. Simple reversal of the well known fermentation equation

$$C_6H_{10}O_5 \cdot 3H_2O \longrightarrow 2C_2H_5OH + 2CO_2 + 2H_2O$$
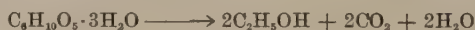
is not acceptable because of the observed R. Q. values in experiments involving ethanol assimilation. The following equation is more compatible with the R. Q. values.

$$C_2H_5OH + O_2 \longrightarrow 1/3 \left(C_6H_{10}O_5 \cdot \frac{25}{24} H_2O\right) + \frac{71}{72} H_2O \quad \Delta F°_{298} = -85,134 \text{ cals.}$$
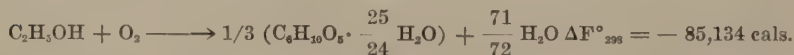
Under our experimental conditions, $\Delta F_{298} = -78,720$ cals. Similar calculations for the direct conversion of ethanol to fatty acids yield ΔF_{298} values which are negative.

In view of the above, one might adopt the attitude that conversion of ethanol to protoplasmic constituents is an energy yielding process and no calculation of the per cent of available free energy captured is possible. In view of the known facts

about metabolism, we have adopted a different approach to this problem.

The escape from the above dilemma is provided by the experimental observation that acetic acid appears transiently in the course of metabolism of ethanol by *Chilomonas*. We have therefore adopted the convention of equating the work done to that required to convert a fraction of the generated acetate to starch or fat. The energy potentially available is that from the oxidation of a fraction of the ethanol to CO_2 and H_2O plus that from oxidation of the remainder to acetic acid. We wished to inquire particularly into the question of whether this latter fraction of the energy was available for metabolic work.

From the data in table 4 assuming only starch to be synthesized we find that 12.8% of the ethanol was oxidized completely. The per cent assimilated (and oxidized to acetate) was 87.2. The fraction of the available energy required for synthetic purposes is therefore

$$\frac{.872 \times 31,758 \times 100}{.872 \times 110,676 + .128 \times 316,465} = 20.2\%$$

Taking into account both starch and fat synthesis (table 4) this percentage becomes

$$\frac{(.668 \times 31,758 + .157 \times 92,600) \times 100}{.825 \times 110,676 + .175 \times 316,465} = 24.4\%$$

If one assumes that the energy obtainable from oxidation of the ethanol to acetate is not available for the work of synthesis these percentages become 68.4 and 64.6 respectively. These are exceedingly high. On the other hand assuming this energy to be available leads to percentages very similar to those found when acetate is the substrate.

DISCUSSION

The results presented in the foregoing sections support and extend the work of Hutchens et al. ('48). These authors estimated 45% of added acetate to be oxidized in the presence of ammonia (i.e. when starch, fat, and protein were being syn-

thesized) and the present experiments indicate 54–57% oxidation. Hutchens et al. based their calculations on oxygen consumption. The R. Q. reported by them for such experiments averaged 1.06. On this basis their figure becomes ca. 48%. The fact that no allowance could be made in the present experiments for the heat cost of protein synthesis leaves open the possibility that our estimate of the acetate oxidized is too high. For the present, therefore, the two sets of experiments may be taken as essentially confirming each other.

Perhaps the most significant finding of the present study is that the fraction of acetate assimilated increases when no nitrogen source is available to *Chilomonas*. Whether this indicates that protein synthesis is more costly or less efficient cannot be said at present. The finding serves as a warning, however, that assimilation studies in which protein synthesis is eliminated are doubtful measures of the assimilation ratios to be expected during growth involving synthesis of protein.

The studies in which ethanol was used as a carbon and energy source indicate that the energy available from oxidation of ethanol to acetic acid is used for reactions of synthesis. Otherwise the fraction of available free energy required seems impossibly high. High energy phosphate bonds apparently can be generated in the course of hydrogen transport (Friedkin and Lehninger, '48). Since such phosphate bonds appear to be the chief form of energy needed for the synthetic reactions involved, use of the energy released in the conversion of ethanol to acetic acid appears reasonable.

In calculating work done in fat synthesis, we have followed the formalistic scheme commonly used in calculating heats of reaction, i.e., showing oxygen produced. This yields what is probably the maximum energy required for synthesis. Perhaps more realistic from the metabolic standpoint is a formulation in which H^+— electron pairs are reserved in other conversions for the reduction of intermediate compounds during fat synthesis. Thus to provide for conversion of a mol of acetate to triolein we reserve 3.23 H^+— electron pairs from the oxidation of another mol of acetate.

Under our conditions:

$$CH_3COO^- + H^+ + 1.19O_2 \longrightarrow 2CO_2 + .385H_2O + 3.23H^+ + 3.23e$$
$$\Delta F_{293} = -140,296 \text{ cal.}$$

and

$$CH_3COO^- + 4.23H^+ + 3.23e \longrightarrow 2/57 \text{ triolein} + 1.79H_2O$$
$$\Delta F_{293} = +26,340 \text{ cal.}$$
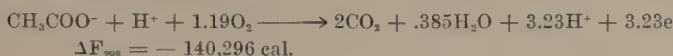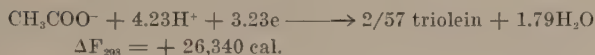
The net effect of these changes is to lower the calculated efficiency somewhat. More detailed knowledge of intermediary fat metabolism is required before a proper scheme can be selected.

SUMMARY

1. Excess heat produced when measured amounts of acetate or ethanol were added to cultures of *Chilomonas* paramecium was measured using a differential microcalorimeter.

2. The observed heat productions were used as a basis for calculations of fraction of substrate oxidized and fraction assimilated.

3. With no nitrogen source present, so that starch and fat were the only products of synthesis, *Chilomonas* oxidized 40.2% of added acetate. The syntheses achieved require 31.3% of the available free energy.

4. When a nitrogen source was present, 56.7% of added acetate was oxidized. Utilization of only 17.8% of the available free energy can be accounted for under these circumstances.

5. Measurements in the presence of a nitrogen source with protein being synthesized are in doubt because of lack of knowledge of either the heat cost or free energy cost of protein synthesis. It seems likely that a smaller fraction of the acetate is assimilated in the presence of a nitrogen source. Whether the fraction of available free energy utilized is really decreased cannot be said.

6. Problems raised by the fact that ethanol can be converted to starch and fat with a net free energy yield are discussed. The convention is adopted that the work done is that required to synthesize starch and fat from acetate generated from ethanol.

7. In the absence of a nitrogen source 17.5% of added ethanol is oxidized completely. Assuming the remainder to be oxidized to acetic acid from which starch and fat are synthesized leads to a value of 24.4% of available free energy utilized for synthesis.

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CYTOLOGICAL AND RESPIRATORY EFFECTS OF CYANIDE ON TISSUE CULTURES [1]

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TEN FIGURES

Although the processes of cell growth have been morphologically analyzed and described, there has been little attempt to correlate such cytological evidence with metabolic activity. Some work has been done on the metabolism of tissues *in vitro*. Warburg and Kubowitz ('27) investigated the metabolic activity of heart fibroblasts in tissue culture finding that the Q_{O_2} remained constant after the 4th transplantation. An inverse relationship between aerobic growth and lactic acid production in tissue cultures was observed by Demuth and Meier ('29). Lipmann ('33) determined that growth is the greatest between the 19th and 43rd hour of cultivation. The high aerobic glycolysis of proliferating tissue cells in cultures was noted by Laser ('33) and others. Zamecnik ('41) calculated that the oxygen consumption per hour of one fibroblast at 22°C. was about 5×10^{-7} μ.

Hydrocyanic acid has long been known to inhibit cellular respiration. Robbie ('46) demonstrated that the effective experimental concentration of hydrocyanic acid can be accurately controlled. Working with sea urchin eggs Krahl, Keltch, and Clowes ('40) and Robbie ('46) noted that concentrations of cyanide which caused a 64% inhibition of res-

piration stopped cellular division completely. The dependence of the *Echinarachinus* egg on aerobic metabolism for cleavage was demonstrated by Robbie ('48).

This study attempts to correlate the inhibition of respiration of tissue cultures by cyanide with changes in cellular activity.

MATERIALS AND METHODS

The problem was divided into two experimental sections: (1) The determination by cytological examination of the effect of various concentrations of HCN on different tissues. Continuous exposure, exposure after 24 hours of growth and exposure for shorter periods with subsequent recovery were studied. (2) Respiration studies were made to determine the relative oxygen consumption in cubic millimeters per hour of cultures under control and experimental conditions.[2]

As a survey of different tissues was desired, heart, spleen, liver and ocular tissues of embryonic chick and newborn rat were cultured.

The medium was composed of equal parts of embryonic extract from 7-day chicks and cockerel plasma in which 1000 units of penicillin G per milliliter was incorporated. The supernatant consisted of 70% cockerel serum, 15% Tyrode's solution and 15% embryonic extract in Tyrode's solution in a concentration equivalent to 15% of embryo juice. The cultures were grown at 37.5°C. for from 48 hours to 7 days.

Cultures for cytological examination. A modified roller tube technique was used for culturing the tissues (fig. 1). Instead of the usual method of lining the inside surface of the test tube, the plasma coagulum containing the tissue was formed on a narrow coverslip, 11 by 22 mm, which was placed in a test tube containing 1.5 cm^3 of supernatant. This procedure was used to avoid inconvenience and impossibility of keeping roller tube cultures for permanent records.

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[2] Q_{O_2} are not calculated for the value would probably be only an approximation of the respiratory quotient.

In cyanide experiments, 0.25 cm^3 of Ca(CN)$_2$-Ca(OH)$_2$ (Robbie, '45) was absorbed on filter paper in the glass stopper well. In 20 minutes the HCN equilibrium between the stock well mixture and the experimental fluid was established. The
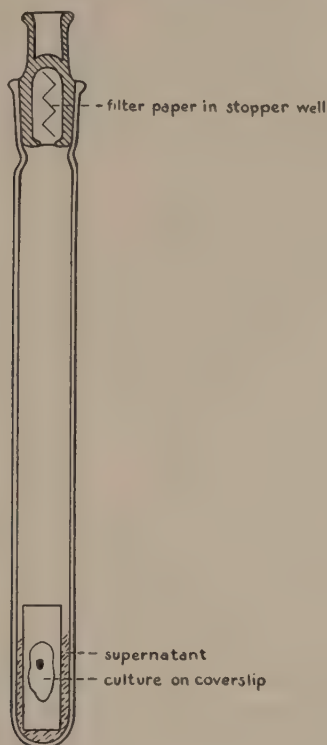
Fig. 1   Modified roller tube for culturing tissues. Plasma coagulum containing tissue formed on coverslip which was placed in test tube containing 1.5 cm^3 of supernatant. Ca(CN)$_2$-Ca(OH)$_2$ absorbed on filter paper in the stopper well.

concentration of HCN in the experimental fluid remained constant throughout the experimental period. Table 1 lists the concentrations of Ca(CN)$_2$-Ca(OH)$_2$ used as stock solutions and the resulting molarity of HCN in the manometer fluid.

When the HCN was removed during the course of an experiment the stock solution was withdrawn, the culture tube

washed thoroughly with Tyrode's solution, and fresh super-
natant added.

At the end of the experimental period the cultures were
washed for 10 minutes in Tyrode's solution at 37.5%C. and
were fixed in 10% formalin for 12 hours. After being washed
thoroughly for at least 7 hours in distilled water the cul-
tures were stained in a weak solution of Delafield's hematoxy-
lin (4 drops in 5 cm³ of distilled water) for 12 hours. After
this they were dehydrated and mounted in balsam.

TABLE 1

Stock solution $Ca(CN)_2 \cdot Ca(OH)_2$ (M.)	Concentration of HCN in experimental fluid (M.)
.65	$.20 \times 10^{-2}$
.313	$.83 \times 10^{-3}$
.163	$.39 \times 10^{-3}$
.081	$.20 \times 10^{-3}$
.0375	$.82 \times 10^{-4}$
.0188	$.38 \times 10^{-4}$
.009	$.18 \times 10^{-4}$
.00413	$.76 \times 10^{-5}$
.00225	$.40 \times 10^{-5}$
.00125	$.22 \times 10^{-5}$
.0005	$.88 \times 10^{-6}$

Cultures for respiration studies. In figure 2 is shown the
3 cm³ Warburg flask used in the respiration studies. The
culturing technique was identical to that used for the roller
tube cultures. One-half cubic centimeter of supernatant
was added to each culture. The flasks were shaken at 60 cycles
per minute in a constant temperature bath at 37.5°C. Each
flask contained 4 pieces of tissue and was gassed with room
air for 10 minutes at the onset of the experiment and at the
end of every 24 hour period. In inhibition experiments the
side bulbs held a solution of $Ca(CN)_2 \cdot Ca(OH)_2$ whereas dur-
ing control periods a 10% solution of $Ca(OH)_2$ was placed in
the reservoirs. When desired the cyanide exposure was ter-
minated by removing the $Ca(CN)_2$ and replacing it by

$Ca(OH)_2$. Following this air was passed through the set-up for 10 minutes. This precaution need not be taken for the 10% $Ca(OH)_2$ in the center well will rapidly absorb the cyanide gas given off by the experimental fluid (Robbie and Leinfelder, '47).
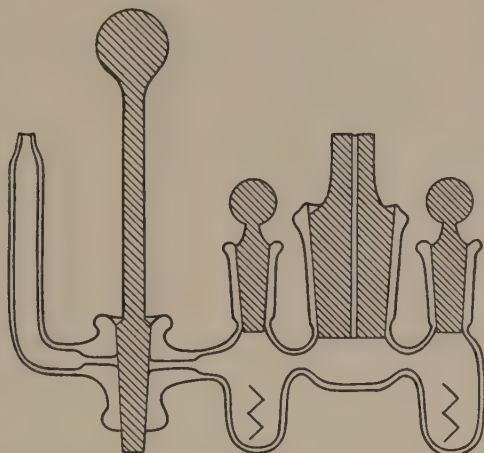
Fig. 2 Three cubic centimeters Warburg flask used in respiration studies. Plasma coagulum containing tissue placed in shallow center well.

EXPERIMENTAL RESULTS

Effects of cyanide on culture growth

The effects of various concentrations of HCN on cellular activity of different tissues were observed by the following methods:

Constant treatment. The curves in figures 3 and 4 indicate the effects of cyanide concentrations which partially or completely inhibit cellular migration for various tissues. Total inhibition occurred in all tissues tested. Tissues inhibited during the first 24 hours of cultivation fell into two classes: (1) Some (9-day chick cornea and iris, newborn rat heart, cornea and retina) show an abrupt breaking point from relatively little curtailment to complete inhibition. This appears to indicate that at a certain crucial concentration cellular activity is profoundly affected. If a gradual inhibitory process
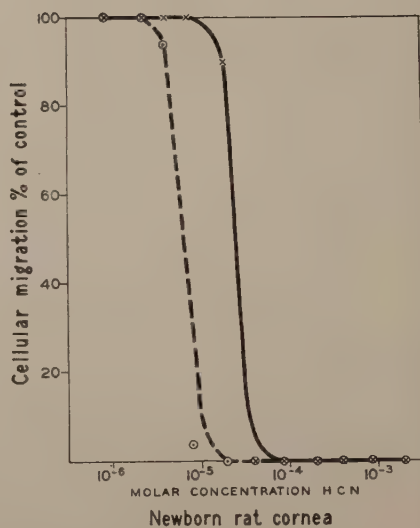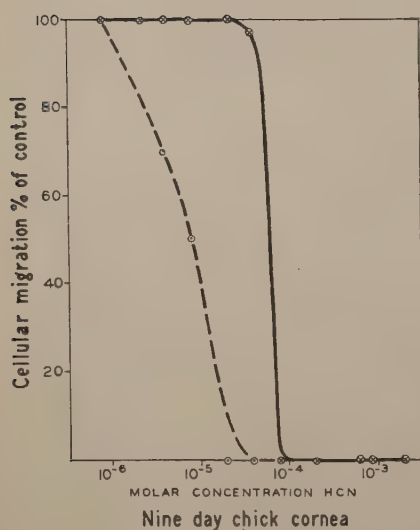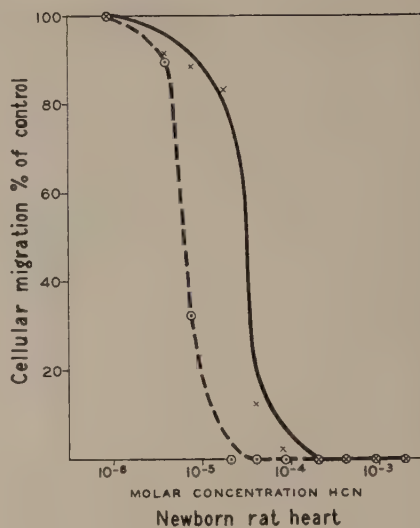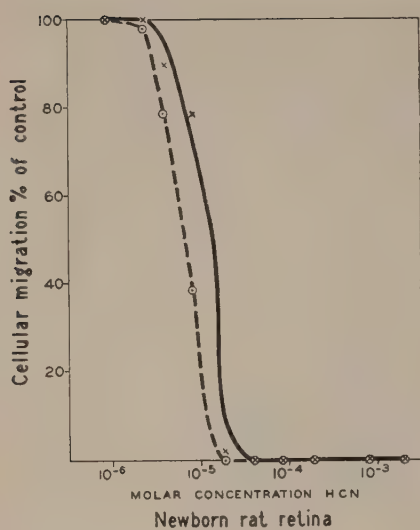
Fig. 3   (See legend on facing page.)
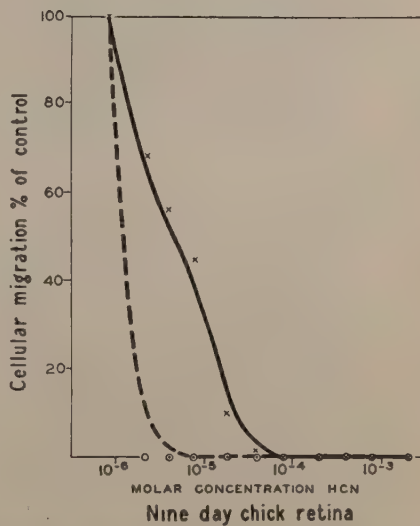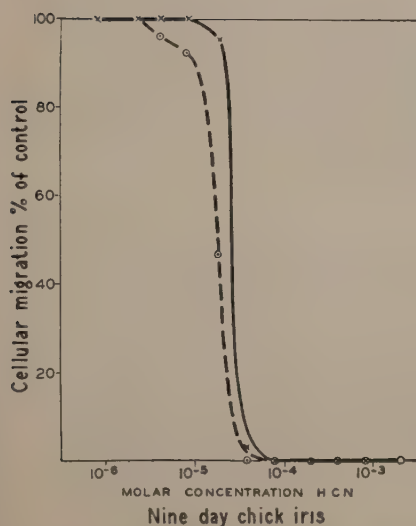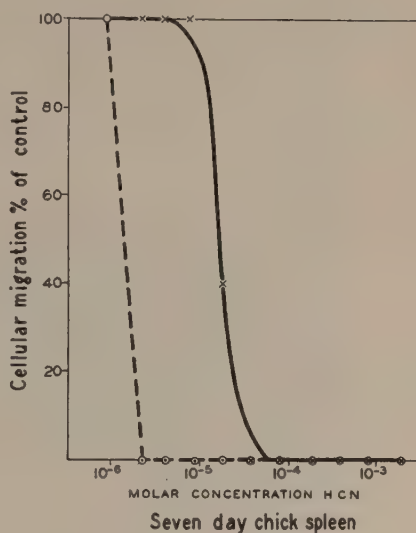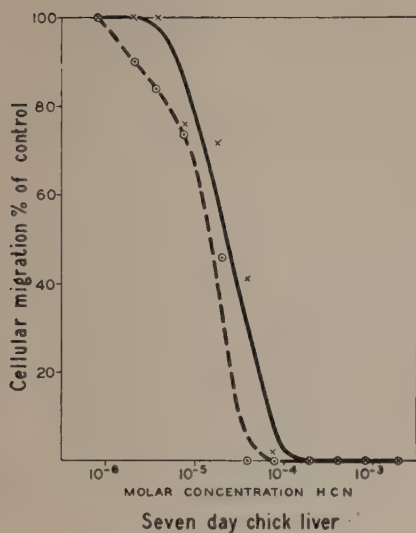
Fig. 3 The effect of cyanide on cellular migration of embryonic chick and newborn rat tissue cultures. Solid lines indicate constant treatment for 24 hours; broken lines, constant treatment for 48 hours. Ordinate shows cellular migration, per cent of untreated control. Abscissa states molar concentration of HCN.

is present the condition of the cultures as determined by counts of cell migration does not reflect that the HCN concentrations used will induce partial inhibition. (2) Others (7-day chick heart and liver, 9-day chick retina, and 14-day chick spleen) show gradual curtailment of activity. They appeared to be sensitive to concentrations of HCN that produced little effect on the other tissues tested.
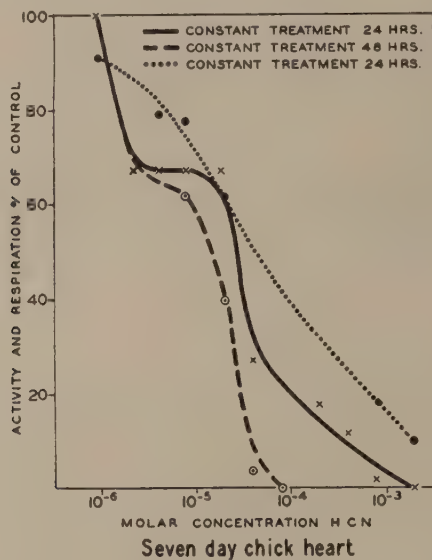
Fig. 4 Effect of cyanide on cellular migration and respiration rate of 7-day chick heart tissue cultures. Cellular migration: solid line, constant treatment for 24 hours. Broken line, constant treatment for 48 hours. Oxygen consumption: dotted line, constant treatment for 24 hours.

Figure 5 gives a pictorial view of 7-day chick heart cultures which have been partially inhibited, completely inhibited, or unaffected by a series of varying concentrations of HCN. At $.88 \times 10^{-6}$ growth is similar to the control; between $.22 \times 10^{-5}$ and $.10 \times 10^{-4}$ activity is curtailed about 33%. Between the latter concentration and $.38 \times 10^{-4}$ there is a sharp increase in inhibition to approximately 73% of the control growth. Complete inhibition for 7-day chick heart

occurs at approximately $.20 \times 10^{-2}$. In such cultures there is no indication of cellular migration. Of course, this does not give any indication of the activity of the cells in the transplant. As shown in figures 3 and 4 fibroblasts of both chick and newborn rat are active at concentrations when the other tissues tested were totally inhibited.
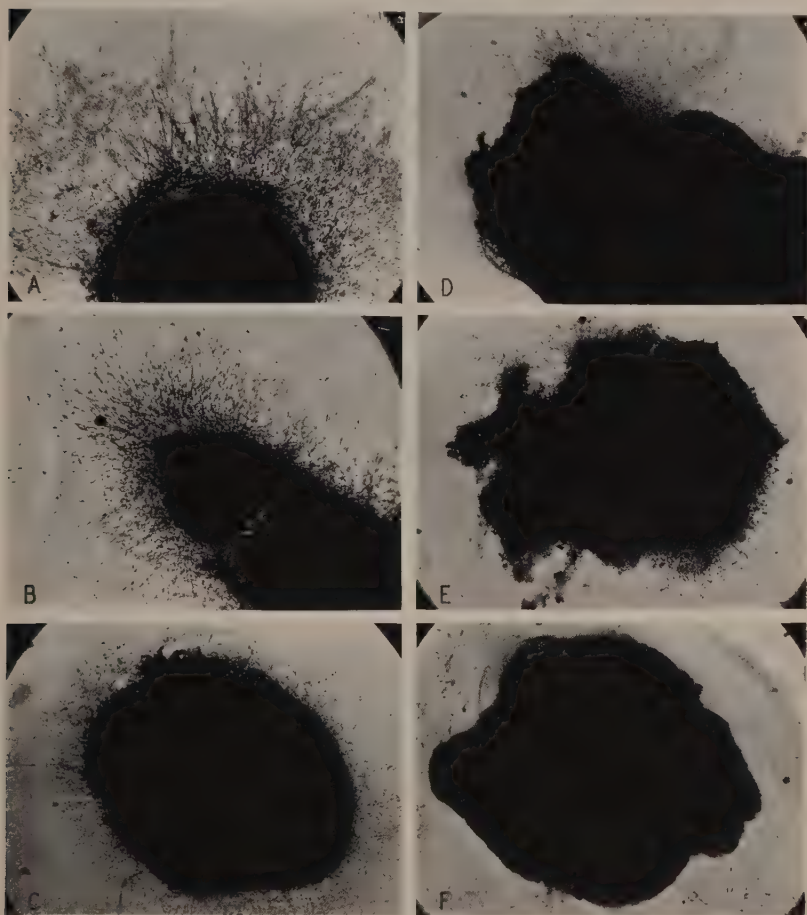
Fig. 5 Photomicrographs of 7-day chick heart cultures which have been unaffected, partially or completely inhibited by constant exposure to various concentrations of HCN for 24 hours of cultivation. a. Untreated control. Treated cultures exposed to various M concentrations of cyanide. b. $.88 \times 10^{-6}$. c. $.18 \times 10^{-4}$. d. $.20 \times 10^{-2}$. e. $.83 \times 10^{-3}$. f. $.20 \times 10^{-2}$.

The number of nuclear abnormalities such as bilobed cells
(fig. 6 a and b), binucleated cells, and fragmented nuclei
(fig. 6 c) increase as inhibition occurs. In cultures of 7-day
chick heart the incidence of bilobed nuclei (fig. 7) increase
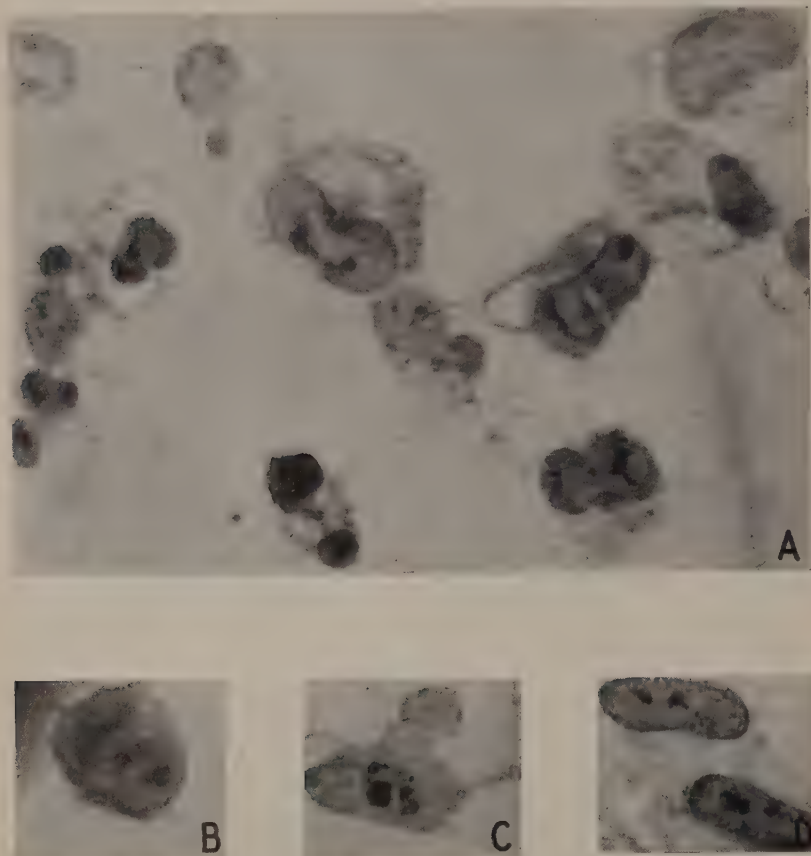
Fig. 6 Photomicrographs showing nuclear abnormalities occurring in 7-day
chick heart cultures treated with HCN. a. Field of a culture treated with
.82 × 10⁻⁴ M. cyanide for entire 24 hours of cultivation showing high occurrence
of bilobed nuclei. b. Bilobed nuclei from some culture showing the failure of
the nucleus to complete division. c. Fragmented nucleus from a culture exposed to
.20 × 10⁻³ M. HCN for entire 24 hours of cultivation. d. Nuclei having very granu-
lar nucleoplasm in a culture grown for 24 hours under control conditions and then
treated with .88 × 10⁻⁶ M. HCN.

as cellular migration is curtailed. Simliar evidence is presented in figure 9 for 7-day chick cornea and retina. The cells appear to undergo normal division to the final stages of teleophase when the nucleus fails to divide. The appearance of binucleated cells indicates that nuclear division occurred without subsequent cytoplasmic division. The migrated cells
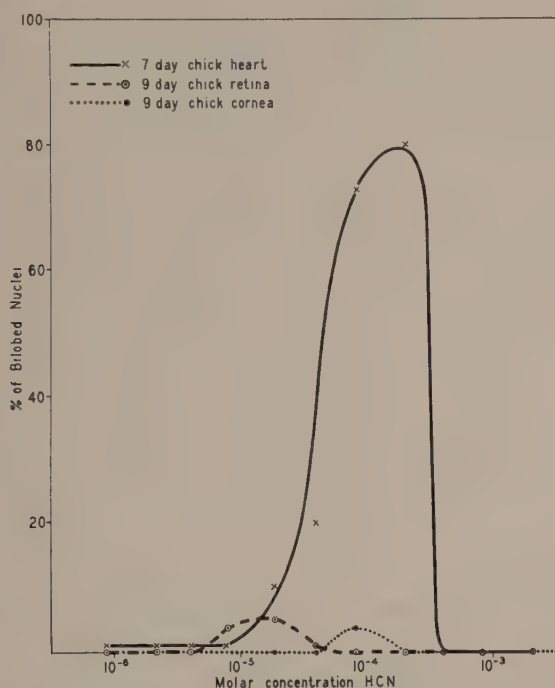
Fig. 7   The occurrence of bilobed nuclei in chick heart cultures treated with various concentrations of HCN constantly for the 24 hour culture period.

in cultures inhibited more than 85% showed few nuclear abnormalities except for an occasional fragmented nucleus indicating that when cellular activity is almost totally inhibited those cells that migrate into the clot do not undergo mitosis.

Activity after removal of cyanide. Cultures of 7-day chick heart exposed to HCN for the first 24 hours of cultivation were grown under control conditions for the following 6 days.

Cultures whose growth was totally inhibited by HCN during the first 12 or 24 hours of cultivation show no cellular activity during subsequent culturing under control conditions up to 7 days. Cultures which had been inhibited for 12 hours to the extent of 23% had completely recovered at the end of 12 hours of control cultivation showing a cell count comparable to controls. Tissues curtailed similarily for 24 hours do not recover as completely or as rapidly (table 2) but by 72 hours appear similar to controls. Table 2 shows the rate of recovery for cultures undergoing partial inhibition.

TABLE 2

The reversibility of inhibition of cellular migration in 7-day chick heart cultures by various concentrations of HCN for 12 hours

HCN conc. of exp. fluid (M.)	% inhibiton after 12 hours	% inhibition after removal of cyanide			
		12 hrs.	24 hrs.	48 hrs.	72 hrs.
$.18 \times 10^{-4}$	23	0	0	0	0
$.38 \times 10^{-4}$	73	79	64	51	
$.20 \times 10^{-3}$	72	71	..	57	
$.39 \times 10^{-3}$	88	83	80	80	..
$.83 \times 10^{-3}$	97	97	96	91	..
$.20 \times 10^{-2}$	100	100	100	100	100

Growing cultures treated with HCN. Cultures of 7-day chick heart grown for 24 hours under control conditions were treated with HCN for from 12 to 24 hours. As shown in figure 8 cells undergo cytolysis at concentrations that do not affect chick heart cultures exposed constantly from the time of culturing. An HCN concentration of $.18 \times 10^{-4}$ caused 33% inhibition in heart cultures constantly exposed to cyanide causes bubbling of the cytoplasm, rounding up of cells, and an overall disruption of cellular activity. The nucleoplasm often appears very granular (fig. 6 d). The reversibility of partial inhibition of activity by HCN at lower concentrations is not seen in cultures exposed to cyanide after 24 hours of control growth. Cells near the clot's surface appear to show

a higher degree of cytolysis than those deeper within the clot.

Respiration studies

To test the validity of the technique oxygen consumption of 2, 4, and 8 pieces of newborn rat cornea was determined.

Seven day chick heart
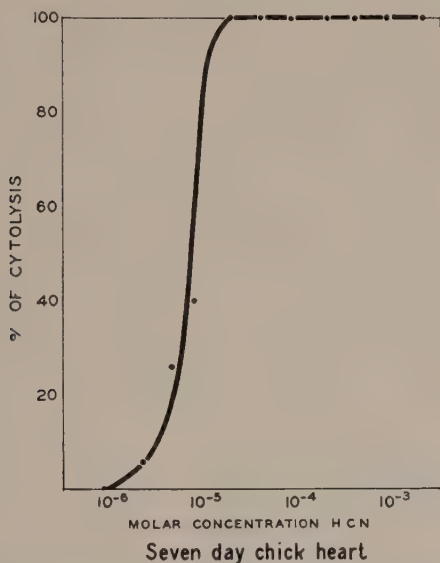
Fig. 8 Cytolysis in 7-day chick heart cultures grown for 24 hours under control conditions and then subjected to various concentrations of HCN for 12 hours.

As shown in figure 9 the method appears to be quite accurate. Absolute accuracy is not necessary because rather profound changes in oxygen consumption are being measured.

Control values for oxygen consumption of 7-day chick heart cultures are listed in table 3. Respiration rates were measured initially, at 24 and 48 hours. Oxygen consumption varied from culture to culture as shown by the ranges indicated in table 3. This variation is in part due to differences in cellular activity as shown by varying degrees of cellular activity. Such variation in control cultures was most pronounced in retina

and least in chick heart. For this reason chick heart was the first tissue to be used as a test tissue for inhibition of respiration by HCN. Experimental values were always compared with controls run at the same time.
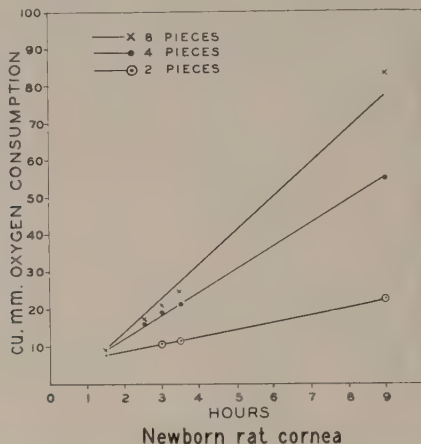
Fig. 9 Oxygen consumption of newborn rat cornea in plasma coagulum culture during the first 9 hours of cultivation. Ten per cent Ca(OH)$_2$ was placed in the side bulbs to absorb the carbon dioxide.

TABLE 3

Oxygen consumption of 7-day chick heart cultures at the time of cultivation, after 24 and 48 hours of growth. Ten per cent Ca(OH)$_2$ was placed in the side bulbs, to absorb the carbon dioxide

Time of readings	Det.	mm^3 O$_2$/hr.	
		range	mean
Initially	18	7–20	11
24 hours	18	22–41	33
48 hours	7	27–49	35

As a check, cultures were grown for cytological examination and transferred to respiratory flasks only at the time of measuring oxygen consumption. The respiratory rate was similar to those of tissues grown originally in Warburg flasks. This was done to ascertain that the values determined in res-

piration experiments represented the activity of the cultures studied cytologically.

Inhibition of respiration by HCN. In figure 10 are shown data from an experiment which demonstrates the effect of various concentrations of cyanide on the respiration of 7-day
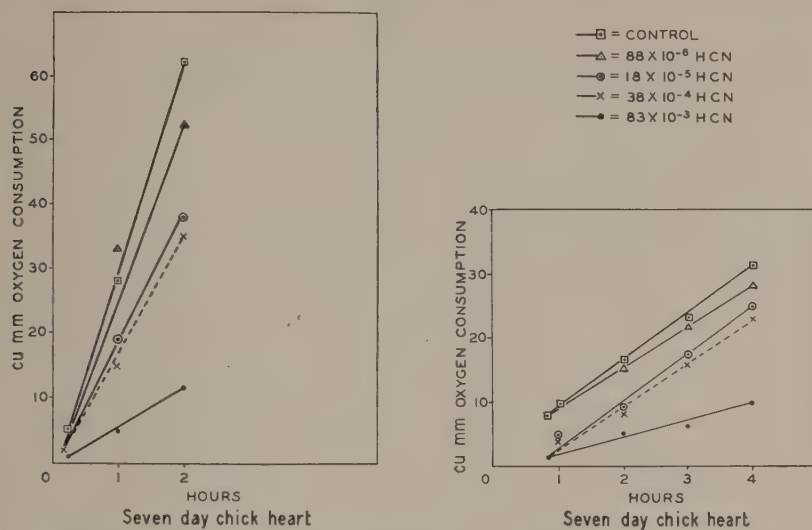
Fig. 10 Effect of various concentrations of cyanide on the respiration of 7-day chick heart cultures initially and after 24 hours of cultivation. Calcium cyanide-calcium hydroxide mixture was used in the side bulbs to absorb carbon dioxide and maintain proper cyanide tension.

TABLE 4

Effects of various concentrations of HCN on respiration of 7-day chick heart initially and after 24 hours of cultivation

HCN con. of exp. fluid (M.)	% inhibiton of oxygen consumption		
	Det.	Initially	24 hrs.
$.20 \times 10^{-2}$	8	85	91
$.83 \times 10^{-3}$	6	61	82
$.38 \times 10^{-4}$	6	53	47
$.18 \times 10^{-4}$	9	40	38
$.76 \times 10^{-6}$	5	17	22
$.40 \times 10^{-5}$	7	8	21
$.88 \times 10^{-6}$	5	0	9

chick heart cultures at the time of cultivation and after 24 hours of cultivation. Inhibition of oxygen consumption by HCN increases from the time of cultivation to 24 hours and then appears to remain constant (table 4). Respiration is never completely inhibited.

DISCUSSION

The preceding experimental data show the dependence of various tissue cells on aerobic metabolism for migration, division and maintenance. The curtailment of migration, the occurrence of nuclear abnormalities and cytolysis as the concentration of HCN increase demonstrate these points.

Cytologically, it is possible to classify the various tissues tested into two groups: (1) those that show abrupt breaking points from relatively little to complete curtailment of cellular migration; (2) those that show gradual curtailment of cellular activity. Cornea and iris of 9-day chick, newborn rat heart, cornea and retina are examples of group 1. Heart and liver of 7-day chick, 9-day chick retina, and 14-day chick spleen illustrate group 2. Tissues in group 1 show a resistance to low concentrations of hydrocyanic acid which definitely cause inhibition of tissues in group 2. For example 9-day chick cornea cultures exposed to $.8 \times 10^{-4}$ M HCN for 24 hours appear like controls whereas 7-day chick heart is curtailed 33% (figs. 3 and 4). This may possibly be a reflection of different maintenance energy requirements.

The inhibition curve obtained for 7-day chick heart indicates that three inhibitory processes may be acting: one at low concentrations of HCN merely curtailing cellular migration; second at higher concentrations inhibiting normal cellular division; and third at highest concentration tested causing death of the cells.

As might be expected longer exposures to concentrations of cyanide inhibit cellular activity to a greater extent. Cultures in group 2 appear to be able to survive prolonged partial respiratory depression with only slight loss in growth

ability (table 2). Evidence for this in tissues of group 1 could not be obtained as only slight or total inhibition was induced.

The variation in sensitivity of 7-day chick heart cultures exposed initially and after 24 hours of growth indicates a possible difference in maintenance energy requirements.

Nuclear abnormalities have been induced by exposing growing cells to urethane (Lasnitzki, '49), cold temperatures (Bucciante, '29), increased acidity (Zweibaum, '35), roentgen rays (Henshaw, '40), and numerous other substances. In this study the normal occurrence of bilobed nuclei was approximately .6%. Kemp ('30) stated that nuclear abnormalities are rare in cultures of normal embryonic chick material for he noted only one cell among 10,000 showing an abnormal mitotic figure. Our control figures are in agreement with Kemp rather than with Stilwell ('44) who reported a 38.9% occurrence of nuclear abnormalities in control chick cultures.[3]

The incidence of bilobed nuclei increased from 20% to 73% when oxygen consumption was curtailed from 31% to 39% of the control value. It is proposed that there may be a range in which oxygen consumption is curtailed to such a degree that mitosis may proceed normally to telophase at which time division is not completed. Such "deeply notched or lobed prophasic nuclei" were noted by Stilwell but no attempt was made to explain their formation. Tyler ('36) was able to induce nuclear division without subsequent cytoplasmic division by low temperature. Robbie ('48) caused incomplete and irregular division in Echinarachinus eggs by a HCN concentration of 10^{-5} M. As mentioned above when cellular activity is almost totally inhibited cells migrate into the clot but do not divide indicating the lack of energy for

[3] The high occurrence of nuclear abnormalities reported by Stilwell may be due to the temperature variation in the incubator (from 37.5 to 42.0°C.). Experimentally she has ruled out the factors of variation in plasma and the photodynamic action of the light bulbs used as a heat source. She concludes that "the physiological state of the cells may in some way be a controlling factor." The experimental variables such as temperature indicate that such cultures cannot be considered as typical controls.

division. The evidence in figure 9 substantiates the view of Tyler ('36) and Robbie ('48) that the maintenance of normal cellular structure and the operation of such functional processes as division call for a certain output of energy.

A morphological description alone is inadequate to analyze the effect of any metabolic inhibitor on tissue growth. The technique for measuring oxygen consumption of tissue cultures described above is similar to that proposed by Lipmann ('32). The values obtained were slightly lower than those obtained by Warburg and Kubowitz ('27). This may be due to the fact that the evolved C_{O_2} was absorbed continuously throughout the experimental period and not at the close of the experiment. Lipmann ('33) reported control values of 32, 28, and 20 mm of oxygen per hour consumed by chick heart cultures. The figures in table 3 fall in this range.

Some correlations between the inhibition of respiration and cellular activity can be made from the evidence derived from experiments on 7-day chick heart cultures. Curtailment of oxygen consumption by 16% for 24 hours does not appear to affect cellular activity. Within the range of 31–39% depression of respiration there is an increase in cellular inhibition from 33–73%. Above 85% curtailment of oxygen consumption there is complete inhibition of cellular migration. After 24 hours there is no further fall in the rate of respiration. The greater sensitivity of the cultures to cyanide of the 48 hours cultivation period is therefore the result of longer exposure (figs. 3 and 4).

It is believed that an attempt to correlate the inhibition of respiration with cytological activity may point out some of the basic interrelationships between cellular activity and metabolism.

SUMMARY

This study attempts to correlate the inhibition of respiration of tissue cultures by cyanide with changes in cellular activity.

Cytological studies

1. A modified roller tube technique for culturing tissues exposed to HCN is described. Heart, spleen, liver, and ocular tissues of embryonic chick and newborn rats were cultured by this method.

2. Cytologically these cultures showed that cellular migration could be unaffected, partially or completely inhibited by various concentrations of HCN.

3. Some evidence for the reversibility of cyanide inhibition is presented.

4. Growing chick heart cultures exposed to cyanide undergo cytolysis at concentrations that do not affect chick heart cultures exposed constantly from the time of culturing.

5. In cultures of 7-day chick heart, 9-day chick cornea and retina exposed to HCN for the entire 24 hours of cultivation the incidence of nuclear abnormalities such as bilobed nuclei, binucleated cells, and fragmented nuclei increase as cellular migration is curtailed between 30% to 80%. Few nuclear abnormalities occur if cellular activity is curtailed more than 80%.

Respiration studies

1. A technique for measuring oxygen consumption of tissue cultures under control and experimental HCN conditions is described. Test experiments to check the validity of the method are given.

2. Data on the inhibition of oxygen consumption of 7-day chick heart cultures by various concentrations of HCN at the time of set-up and after 24 hours of cultivation is presented.

Some correlations between the inhibition of respiration by cyanide and cellular activity in tissue cultures are proposed.

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THE MAMMALIAN BREATHING MECHANISM

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Renewal of the gaseous medium in contact with respiratory epithelium of air breathing animals involves two basic physical considerations: namely (1) mechanisms to induce volume flow of air in and out of the respiratory tract, and (2) mechanisms to regulate molecular diffusion of the gaseous components of air at the respiratory epithelium. Development of the alveolar lung in vertebrates has been recognized as important not only for the large surface area involved, but also for the effective mechanism provided to maintain relatively constant physical conditions for diffusion in the presence of gross cyclic flow patterns in the breathing exchange. The effectiveness of CO_2 as a respiratory regulator, for example, appears to depend fundamentally on such a mechanical buffering action. These considerations of respiratory exchange in vertebrates have become complicated by also subserving the accessory functions of olfaction and in many species, temperature regulation.

Certain elements of the mechanism for volume flow in terrestrial mammals have been carefully studied, particularly the rhythmical breathing cycle and aspects of exchange such as predominate in man (e.g. Guyton, '47). There has been failure to recognize certain intrinsic features of the process which are characteristic of other terrestrial mammals. These features are (1) polyphasic patterns in the breathing cycle and (2) rhythmically recurring accessory cycles. Such unusual breathing patterns, like those of aquatic mammals (Scholander, '40),

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provoke interesting questions about the mechanisms for diffusion. A consideration of these breathing factors is the subject of this report.

<center>MATERIALS AND METHODS</center>

Horses *(Equus caballus)* and albino rats *(Rattus norvegicus)*. which can be taken to represent extremes of mammalian size, were chosen for detailed study. Two horses, 16 rats, and one guinea pig for experimental study were selected after preliminary observation periods to eliminate respiratory abnormalities which are common to these animals, especially emphysema in horses and respiratory infections in rats. Other specimens and species (rabbit, cat and dog) to be mentioned were used as soon as received from suppliers, but they had no obvious respiratory disorder.

It was found important to isolate horses for at least two hours and to permit adjustment to experimental procedures if basal conditions were obtained. The eupneic breathing pattern was especially sensitive to disturbances. Pneumographs were used to study superficial breathing movements. Intra-nasal pressure was recorded through a catheter in the nasal meatus, the intra-tracheal pressure through a 16-gauge needle inserted near the base of the neck, the intra-pleural pressure through a blunt trochar inserted through a 15-gauge needle between the 7th and 8th ribs mid-laterally, the intra-abdominal pressure as for intra-pleural pressure with the trochar in the left flank region, and intra-rectal pressure from a balloon. Procaine was used for the insertion of needles.

Rats were observed in their cages under basal, undisturbed conditions for information about the rate and character of breathing cycles, and also three experimental procedures were developed to analyze rat breathing; namely (a) manometric and volumetric readings and tambour recordings from a closed chamber containing the whole animal, (b) spirometric readings and tambour recordings from a closed chamber which included

the head only, and (c) tambour recordings from a thoracic pneumograph.

The closed animal chamber apparatus for small mammals is shown in figure 1. The chamber was made from a common glass preserving jar (two quart) into the metal reinforced cap of which copper tubes were soldered. With the paraffine filler used to reduce volume and increase sensitivity, the chamber provided a respiratory volume of about 1300 ml. Flexible connections were made of fiber reinforced rubber pressure
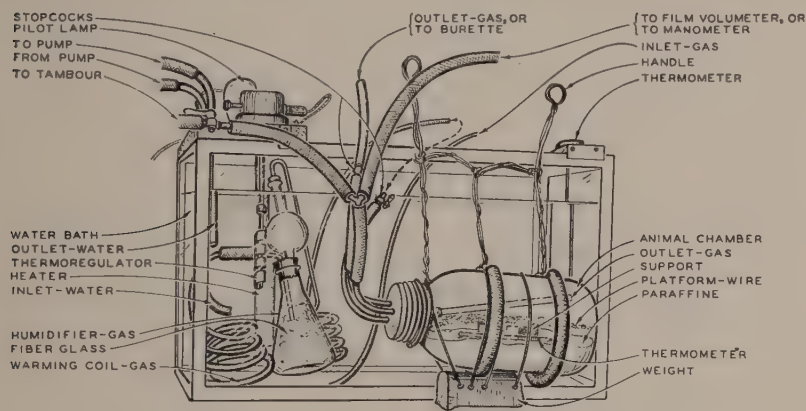
Fig. 1   Closed respiration chamber apparatus to study differential effect on air volumes of breathing movements and airway resistance.

tubing of minimum length which eliminated expansion error over the pressure range involved (up to 3.0 cm of water). For gas volume changes in the chamber, a large tambour (diam. 7.5 cm), with low membrane resistance in relation to volume change over the range of excursion employed, recorded through a thin aluminum disk supporting a light cork pedestal; the pedestal included a razor blade chip which acted against a very light reed stylus. The magnification was about 72 ×. The tambour was standardized for measurement of volume and pressure changes in the chamber by adding water from a

burette and determining pressure result with a water manome-
ter on a micrometer scale. A direct volume change indicator
was also used, a film volumeter, which will be described in con-
nection with the spirometer. The animal rested in the chamber
on a wire mesh platform below which a small pool of acidulated
water was placed to humidify without CO_2 absorption. CO_2
absorption was not used in any of the closed chamber experi-
ments, including the spirometer, in order to avoid analysis of
the variable absorption rate. Rather, the length of time the
specimen could be enclosed without disturbing increase in CO_2
or anoxia developing was calculated from O_2 consumption data
(Moses, '47) and runs were scheduled accordingly with ade-
quate intervening periods to flush the chambers.

Normal rats, conditioned to handling, readily entered the
animal chamber. At most of the temperature ranges used
(about 28°C.) and in bright light they soon became quiet and
often asleep over periods of three to 5 hours with only occa-
sional interludes of cleaning and investigative activity. Basal
conditions were therefore readily attained, and it is for this
state in particular that the discussion to follow applies. How-
ever, some specimens were placed in the chamber under ure-
thane or pentobarbital anesthesia for comparison with those
used in the spirometric procedure in which all specimens were
necessarily under anesthesia from the outset.

Rats which had a tracheal or esophageal cannula in position
were also studied in the animal chamber. The operation for
insertion of a cannula was performed under injection anes-
thesia to avoid complication of the volatile anesthetics in
volume measurements. The tracheal cannula was inserted
lungward in the upper one-third of the trachea and fastened by
sutures to the skin of the neck after the incision was closed.
The dimensions of the cannula were selected so as to approxi-
mate the resistance and dead space of the portion of the tract
thus by-passed. The esophageal cannula was inserted mouth-
ward in the upper one-third of the esophagus, and the distal

end of the esophagus was ligated. These rats were placed in the chamber while still under anesthesia, and their breathing was studied during recovery.

The spirometric procedure involved two major difficulties. The first problem was to encompass the head and neck in a pressure tight enclosure with a minimum of flexibility and without interference with normal breathing by restriction of the trachea or circulation. The second problem was to provide a volume indicator of sufficient freedom from frictional damping and inertia to follow the volume changes of a single breathing cycle.

The apparatus with which spirometric studies were made is shown in figure 2. It consisted of two concentrically arranged
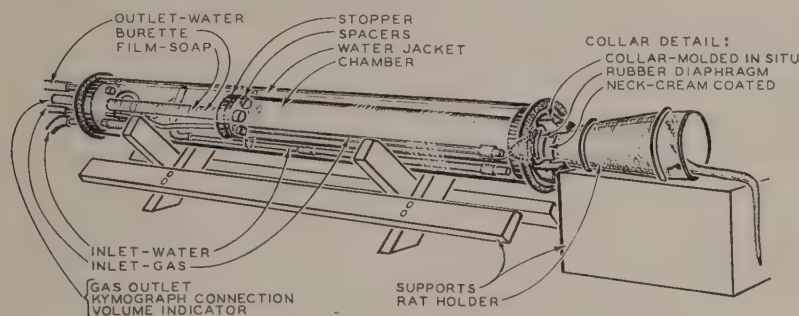
Fig. 2   Film spirometer apparatus to study air volumes involved in breathing. Neck enclosure cut away to show detail.

glass cylinders, horizontally placed, the inner of which was a respiration chamber (diam. 4 cm, length 40 cm) that continued into a 25 ml burette. The outer cylinder — from one end of which the end of the respiration chamber protruded and from the other end the burette — served as a water jacket for regulation of gas temperature by means of circulation from the constant temperature bath. The chamber provided a respiratory atmosphere of about 500 ml, and it contained a thermometer and a gas inlet tube near the open (head) end. The burette served for volume measurements and gas outlet, and the re-

cording tambour was connected to it. Inflowing gas could be saturated and temperature controlled as in the animal chamber experiments.

The problem of enclosing the head in the chamber was solved with a "built-up" seal shown in a cut section on the diagram. A diaphragm of rubber dental dam was fastened over the flanged end of the spirometer tube, and a hole was made in the diaphragm closely following and somewhat smaller than the cross-sectional outline of the specimen's neck. This hole was adjusted to a snug but physiologically non-restricting closure with the rat in place. A pool of acidulated water was placed in the bottom of the spirometer chamber. The rat was prepared by anesthetizing, trimming vibrissae and coarse hair on the neck; heavily coating the entire area from ears to shoulders with a thick cream (DuPont Pro-Tek and glycerine) to eliminate air space of the hair coat; and placing it in a glycerine coated, tapered glass cylinder so that its head and neck protruded beyond the end. The cylinder was large enough to support the body without restriction of breathing movements, and the glycerine coating reduced the effectiveness of body and leg movements. The head was then placed in position through the diaphragm of the spirometer and the hair coating smoothed evenly. A collar-shaped, trough-like rubber form was next filled with a quick-setting, elastic hydrocolloid (D-P dental impression cream) and brought snugly around the neck and flanged end of the spirometer chamber. The open top of the form was filled with the cream, and the entire mass was allowed to set (about one minute) to form a thick, relatively rigid collar. The collar thus enclosed the neck and the end of the spirometer and immobilized the rubber diaphragm.

The problem of a volume indicator was solved by swabbing the spirometer burette with a dilute solution of synthetic soap (Mabex bubble solution) and placing films of the concentrated solution across the lumen. The films were very persistent, lasting as long as two weeks in a stoppered burette. These film-containing burettes were also used in conjunction with the

previously described animal chamber to measure its volume changes; such burettes will be designated *film volumeters*, and the spirometer thus provided will be designated a *film spirometer*.

A pneumograph was devised for the rat from a rubber balloon and soft brass spring. The response of this was reversed from the typical pneumograph, as used for the horse, in that inspiration decreased its volume, and thus the tambour used for recording marked inspiration upward.

Lung volume of the rat was determined by a displacement technic with apparatus shown in figure 3. The rat was lightly
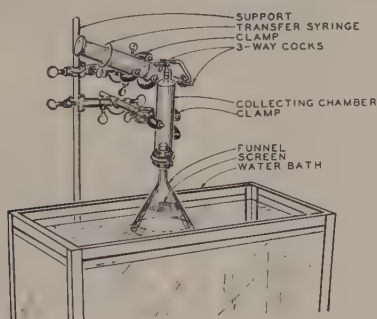
Fig. 3   Apparatus to collect by water displacement the total gas expelled from the respiratory system of a rat for nitrogen analysis.

anesthetized, soaked in water to saturate the fur, its nostrils stopped with a temporary plug of heavy grease in rubber tubing, its mouth held shut and its head immediately placed under the edge of the inverted funnel. Distilled water in equilibrium with room air was used to fill bath, funnel and syringes. All instruments and the operator's gloved hands were freed in the bath of adherent bubbles before being placed under the collecting funnel.

As soon as the rat's head was under the funnel, the plug was removed and all of the terminally expired air collected. Still held under the funnel, the trachea was exposed and

clamped, air in the passages was removed with a probe, and the lung was exposed and removed with the attached length of trachea cut just anterior to the clamp. The lung was floated into a fine-mesh nylon bag, the remaining trachea severed and emptied, and the lung then punctured and thoroughly macer-
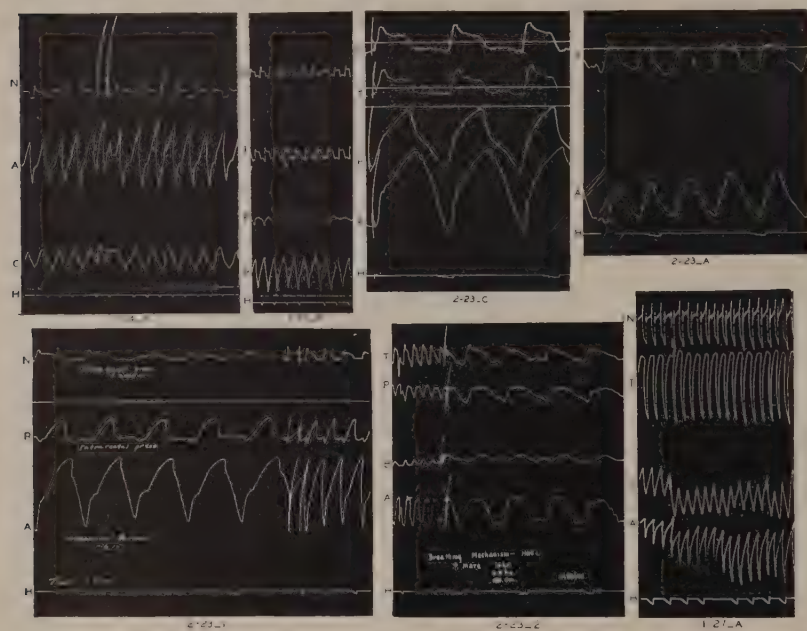
Fig. 4   Records of correlated factors in horse breathing (1–14 — gelding, age 2 yrs., wt. 427 kg, ht. 150 cm) (1–27 and 2–23 — mare, age 10 yrs., wt. 418 kg, ht. 150 cm). N, intra-nasal pressure; A, abdominal pneumograph; C, thoracic pneumograph; T, intra-tracheal pressure; P, intra-pleural pressure; R, intra-rectal pressure; B, intra-peritoneal pressure; H, time (5.0 sec.).

ated until all possible gas was removed and only tissue pulp remained. Small adherent bubbles were freed from the collecting funnel with a rubber policeman, and the entire volume of gas which collected in the chamber was drawn into the transfer syringe. The gas was then transferred to a gasometric pipette (McCutcheon, '43a) and analyzed for nitrogen only.

O_2 and CO_2 were ignored because there was no control over absorption, release, or diffusion in the water and dilute blood involved in the collection. The nitrogen content would remain constant and provide an indicator from which total lung volume could be calculated.

RESULTS

Records of various pressure factors in the breathing cycle of normal horses are shown in figure 4, and a composite dia-
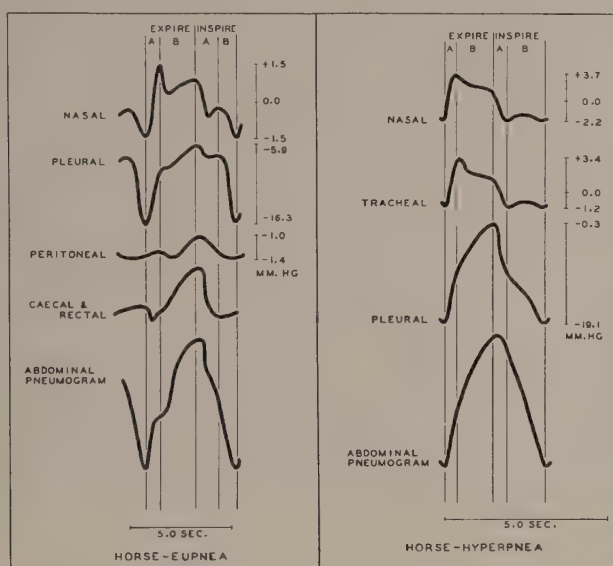
Fig. 5   Composite diagram of factors in breathing cycle of horse, analyzed to show correlation of intra-coelomic and intra-passage pressures with external musculature.

gram of some of these tracings adjusted to the same time ordinate for analysis is shown in figure 5. Reference will also be made to certain atypical conditions shown in figure 4, records 2-23-A and 1-27-A, which respectively show (1) the effect of nasal epinephrine spray and (2) the effect of reclining position.

Points of special interest which are displayed in records of horse breathing are: (1) the normal resting cycle (eupnea) is

clearly polyphasic with dual inspiratory and expiratory phases; (2) thoracic movements are not as great as abdominal movements; (3) with increased breathing (hyperpnea) the dual expiratory and inspiratory phases become obscure in abdominal movements, but they clearly persist in respiratory tract pressures (nasal and tracheal) and in intra-pleural inspiratory pressures; (4) the second pressure peak in expiration is lower than the first in respiratory passages (nasal and tracheal) but higher intra-pleurally, intra-peritoneally, and in intestinal passages (caecal and rectal); an intra-caecal and rectal pressure peak occurs at the end of inspiration which does not reflect a corresponding intra-peritoneal (abdominal) peak; (5) the effect of bronchiolar dilatation by epinephrine is to greatly obscure the dual character of both inspiration and expiration in tracheal pressures and abdominal movements; (6) the effects of restraint in reclining position is to obscure the dual character of abdominal movements and intra-tracheal pressure changes, but to greatly exaggerate the dual character of intra-nasal pressure changes. Intra-tracheal pressure of 18 mm Hg was developed during each of these cycles.

Not shown on the diagram are measurements of the total range of intra-abdominal pressures. These varied from -180 mm H_2O at insertion of trochar to -14 mm at rest. Excitement lowered the pressure to a range of -50 to -80 mm H_2O, which gradually rose to resting level in 10 to 20 minutes after stimuli ceased. The negative values are of interest in view of the report of Rushmer ('46) for intra-abdominal pressures in an erectly supported dog, which indicated that the pressure is a hydrostatic pressure resulting from the overlying mass of movable abdominal organs in relation to the dome of the diaphragm. Measurements as made in the horse would partly reflect local intestinal movements at the tip of the trochar.

Records from studies of rat breathing are shown in figures 6, 7, and 9, and data are given in tables 1 through 4. For comparative purposes, records from one guinea pig are shown in

figure 6. With the discovery of rhythmically recurring acces-
sory cycles in these animals, efforts were made to analyze and
interpret the cycles by the variety of methods previously out-
lined. The accessory cycles show clearly on the records, but
the ventilation cycles are so small that their characteristics will
not be clear in the reproduction of chamber and pneumograph
records. Typical diphasic ventilation cycles have been ade-
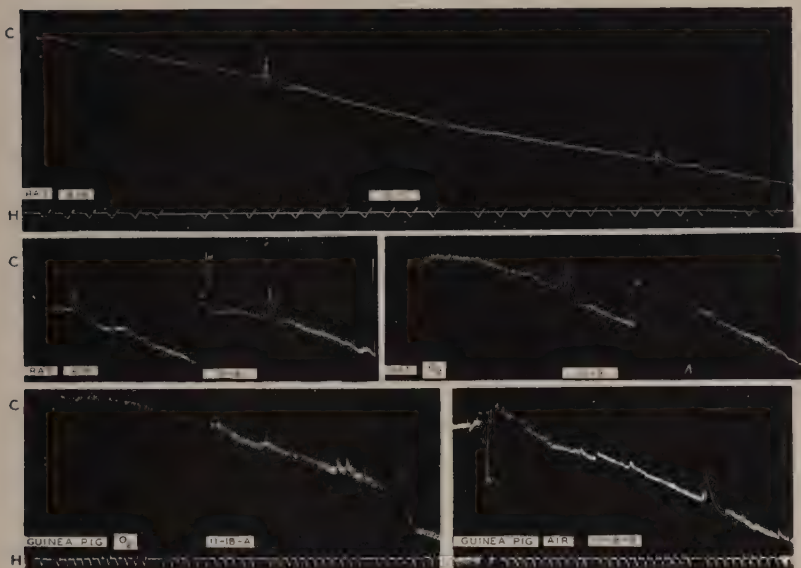
Fig. 6   Records from closed respiration chamber to show ventilation and comple-
mentary breathing cycles in rodents. C, chamber pressure; H, time (5.0 sec.).

quately described by Guyton ('47). Not shown are records of
accessory cycles in rabbit, cat, and dog, which were recorded
by a bell type spirometer through a tracheal cannula from
specimens breathing air and 30% O_2. Knowlton and Larrabee
('46) recorded similar cycles in a cat. No studies of these ani-
mals comparable to studies on the rat have been made. It can
only be said at present about accessory cycles in these animals
that (1) their rhythmical occurrence is established, (2) they

TABLE 1

Ventilation and complementary cycle frequency

Normal animal

EXP. NO.	WT. GM	VENTILATION NO./MIN.	COMPLE- MENTARY NO./HR.	PROCEDURE	GAS	CONDITION	REFERENCE
7–20	273 ♀ rat	53	13–53 26 av.	In cage 27.5°C.	Air	Sleep	Direct count
10–13	353 ♂ rat	..	21	In chamber 26°C.	Air	Rest	Direct count
10–4	273 ♀ rat	60 ..	23–28 55	In chamber 26.5°C. bar. 767.0	Air Air	Rest Activity	Record
10–11	273 ♀ rat	84 66 60	63 49 37	Thoracic pneumograph 30°C.	Air Air Air	Activity Quiet Sleep	Record Exp. 10–4
11–6	322 ♂ rat	60 66–72	20 26–31	In chamber 28.0°C. bar. 760.0	Air Air	Rest — sleep Activity	Record Exp. 11–2
11–9	302 ♂ rat	60 84 60	26 34–69 9	In chamber 28.0°C. bar. 764.0	Air Air O$_2$	Rest — sleep Activity Sleep	Record Exp. 11–8
10–1	253 ♂ rat	54 90	10–19 24–30	In chamber 28.3°C. bar. 772.8	O$_2$ O$_2$	Pentobarbital depression Pentobarbital recovery	Record
10–17	288 ♂ rat	72 78	5–22 33–60	In chamber 26.5°C. bar. 767.9	Air Air	Pentobarbital Recovery from anesthetic	Record
11–18	495 ♀ guinea pig	84	12–22	In chamber 26°C. bar. 757.2	Air	Quiet	Record

TABLE 2

Ventilation and complementary cycle frequency
Anesthetized animal

EXP. NO.	WT. GM	VENTILATION NO./MIN.	COMPLE-MENTARY NO./HR.	PROCEDURE	GAS	CONDITION	REFERENCE
11-12	322 ♂ rat	104	0/0.75	In chamber 26.5°C. bar. 765.3	Air	Urethane, depressed; tracheal cannula; very shallow tidals	Record Exp. 10-17 Exp. 11-2
		...	23		Air-O₂	Continued as above, except tidals deeper	
		0?	60?		Air	Very deep tidals with marked retention; complementary cycles?	
		15	0/0.1		Air	Shallow tidal	Record B
		13	2		Air	Deep tidal	
		90	0/0.75		Air	Very shallow tidal, depressed	
		60	83		Air	Excitation	
		0?	30?		Air-O₂	Biot type	
		0?	48?		Air	Repetition of complementary cycle?	
		66	40		Air	Recovery 6 hrs.	
11-16	272 ♂ rat	90	0/0.25	In chamber 27°C. bar. 759.9	Air	Pentobarbital, depressed; tracheal cannula	Record A
		66	13-46		Air	Recovery from depression	
		138	10		O₂	Quiet	
10-22	283 ♂ rat	30	2-6	Spirometer 28°C. bar. 766.1	Air	Pentobarbital — neck and leg restraint, depressed	Record A
		60	120		Air	Recovery — struggle	
10-31	310 ♂ rat	66	11	Spirometer 28°C. bar. 762.1	Air	Pentobarbital — neck and leg restraint	Record B
		72	110		Air	Recovery — movement	

are CO_2 sensitive in amplitude and frequency, and (3) the frequency diminishes with characteristic increase in species size and decrease in breathing rate. On this latter point, 12-hour intermittent records of horse breathing with continuous recording of as long as two hours at a time give no clear indication of accessory cycles. An opportunity to examine recent unpublished records obtained with the Scholander metabolism apparatus by Dr. Harald Erikson, in a comparative study of respiratory metabolism between Caucasians and Eskimos,
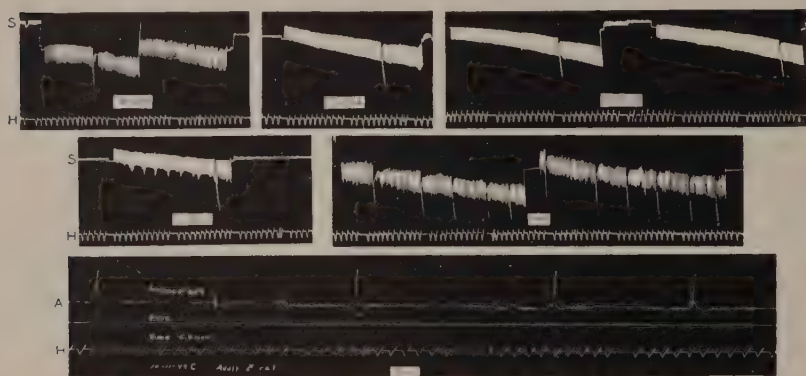
Fig. 7   Records from spirometer and pneumograph to show air movements and breathing movements in cycles of rat. (10–11 pneumograph, rat in normal atmosphere; 10–26 through 11–1 spirometer with air, except 11–2A with O_2; 11–2–C and 11–1 relationship of complementary to ventilation cycles in periodic breathing. See tables for data.) S, spirometer pressure; A, pneumograph; H, time (5.0 sec.).

shows that analogous cycles occur in man in a rhythmical sequence with a resting frequency of about three or four an hour. Similar cycles are a characteristic of breathing in seals as reported by Scholander ('40). In diving animals these cycles are accompanied by closure of the nares.

Returning to the results of rat studies, a composite diagram traced from the separately recorded elements shown on the records is presented in figure 8. These records were adjusted to the same time ordinate for analysis of the events in a com-

plementary cycle, as indicated on the diagram. Important factors entering into the various records should be especially noted, namely (1) slope traced by ventilation cycles in chamber and spirometer, and (2) elements of pressure or volume change involved in the closed chamber. With reference to the slopes traced by the ventilation cycles, such slopes are usually considered in metabolism studies to reflect (1) oxygen consumption in relation to CO_2 output (RQ), or (2) difference be-
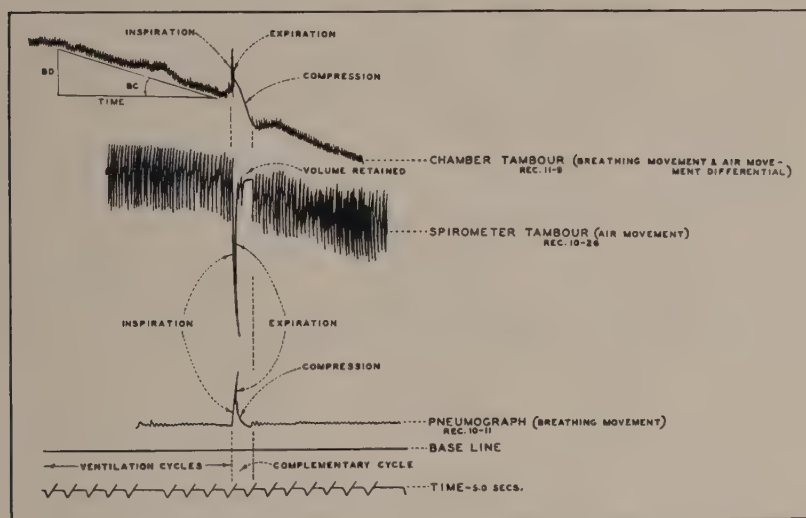
Fig. 8 Composite diagram of factors in complementary cycle of rat, analyzed to show correlation of separately recorded elements.

tween inspired volume and expired volume due to changes in mid-point of breathing movements. The latter is designated RD (respiratory difference) by Scholander ('37), and he emphasizes its importance as a source of error in metabolism studies. Since this difference is measured as a specific breathing difference, it is here designated on the diagram as BD *(breathing difference);* that is, inspired volume minus expired volume. In addition to the factors just mentioned, a further element affecting BD seems apparent on the records, namely

TABLE 3

Complementary cycle volume retention

I. From tambour recordings of chamber gas (O_2)

Data:

EXP. NO.	WT. GM	TEMP. — °C. BAR. — MM HG	CYCLE (a) Distance max.–min. av. mm	CYCLE (b) Time max.–min. av. sec.	BC (c) mm/ min.	CALIBRATION (d) Vol. mm/ml	CALIBRATION (e) Press. mm/mm H_2O	REFERENCE
8–10	207 ♀	30.8 757.9	4.9–3.8 4.0 (12)	10–5.5 7.4	6.8	9.50	5.57	Record
9–14	273 ♀	25.5 761.1	16.0–8.1 10.4 (10)	7.0–5.0 5.7	23.6	15.04	6.69	Record Exp. 7–20 Exp. 10–4 Exp. 10–11

Calculation:

EXP. NO.	(1) VOL. (a/d) MAX.–MIN. AV. ML	(2) BC/CYCLE (bc/60d) MAX.–MIN. AV. ML	(3) PRESS. (a/e) MAX.–MIN. AV. MM H_2O	(4) CYCLE VOL. (1–2) MAX.–MIN. AV. ML
8–10	0.516–0.400 0.421	0.12–0.07 0.09	0.88–0.682 0.718	0.396–0.33 0.331
9–14	1.06–0.54 0.69	0.19–0.14 0.15	2.38–1.21 1.55	1.01–0.41 0.54

II. From film volumeter data on chamber gas

EXP. NO.	WT. GM	TEMP. — °C. BAR. — MM HG.	CYCLE VOLUME MAX.–MIN. AV. ML	BC ML/MIN.	GAS	CONDI- TION	REFERENCE
11–8	325 ♂	28 759.5	0.85–0.1 0.40 (3)	0.73	Air	Rest	Record A–D
11–8	302 ♂	28 756.9	0.70–0.25 0.43 (6)	1.08	Air	Rest	Record E Exp. 11–9
10–4	273 ♀	28 767.0	0.6–0.5 0.52 (5)	...	Air	Rest	Record Exp. 7–20
			0.6–0.45 0.54 (5)	...	O_2	Rest	Exp. 9–14 Exp. 10–11

TABLE 4

Ventilation and complementary cycle volumes
From film spirometer

EXP. NO.	WT. GM	TIDAL ML MAX.–MIN. AV.	CYCLE RETENTION ML MAX.–MIN. AV.	PROCEDURE	GAS	CONDITION — REMARKS	REFERENCE
9-27	235 ♂	1.5–0.4 0.94 (14)	1.7–0.3 0.92 (4)	Spirometer 37°C. room 24°C. bar. 768.6	Air	Pentobarbital — rectal temp. 34°C.	Indicator readings
10-19	283 ♂	2.2–0.6 1.2 (23)	1.4–1.0 1.2 (4)	Spirometer 28°C. room 24°C. bar. 763.3	Air	Pentobarbital	Indicator readings
10-26	293 ♂	2.5–1.9 2.2 (33)	. . . . . .	Spirometer 28°C. room 23°C. bar. 764.9	Air	Pentobarbital recovery, struggle	Record
10-31	310 ♂	1.6–0.7 1.1 (27) 1.9–1.2 1.5 (35) 6.0–3.0 4.7 (15)	. . . . . 1.0–0.5 0.74 (5)	Spirometer 28°C. room 24°C. bar. 762.1	Air Air Air	Pentobarbital — depressed 30 min. later, surgical anesthesia Cycle inspiration magnitude	Record
11-1	334 ♂	2.7–1.4 1.9 (36)	1.1–0.0 0.7 (23)	Spirometer 28°C. room 21°C. bar. 762.2	Air	Pentobarbital, and metrazol	Record
11-2	322 ♂	1.9–1.3 1.7 (50)	1.1–0.2 0.6 (6)	Spirometer 28°C. room 22°C. bar. 760.0	Air O_2	Urethane — regular, uniform breathing — much less variation than under pentobarbital	Record Exp. 10-17 Exp. 11-6

an altered exchange ratio which is independent of midpoint changes but caused by intra-pulmonary retention through pressure alteration. Another factor shown on the composite diagram is the slope of the line reflecting combined respiration and breathing factors. This slope is defined by BD divided by Time, and it is described as a *breathing coefficient* (BC).

Gas volume changes in the closed chamber (figs. 6 and 8) resulted from (1) differential expansion and compression of the total volume of gas within the rat, as opposed to that in the chamber, against the resistance to movement of gas in and out of the chamber; and (2) concomitant RQ factors. If the metabolism factors (RQ) are standardized through the general slope (BC), then any sudden alteration of this slope, too large to be accounted for by alteration in RQ must reflect pressure changes in the rat. Such pressure changes are evident in the diagram as an outstanding characteristic of the complementary cycles, for spirometer records show air retention during the cycle and pneumograph records show no changes in midpoint. Quantitative data concerning the cycles are given in tables 1 through 4.

Certain general features of the complementary cycles are of special interest in the recordings and data; namely (1) variability in degree of compression both in air and in O_2, (2) generally a more uniform and larger compression but lower frequency in O_2 than in air, (3) occasional ventilation cycles may occur during the typical apneic phase of compression, (4) anesthesia (pentobarbital) suppresses the pause (apnea) and the amount of retention becomes more variable (fig. 7), (5) the effect of the compression phase may occur without apnea in a kind of abortive cycle (fig. 6, 11–9), (6) CO_2 or exercise increases amplitude and frequency of complementary cycles in the same manner as ventilation cycles, (7) periodic breathing (figs. 7 and 9) involves both ventilation and complementary cycles in a manner which may involve the suppression of the former and increase of the latter. Other recordings, which are not reproduced, of rats with tracheal cannulae or esophageal

cannulae showed by results similar to those of figure 6 that closure of the glottis or swallowing did not account for these results.

One record from the closed chamber of a rat with a tracheal cannula is presented with analytical remarks (fig. 9) because it
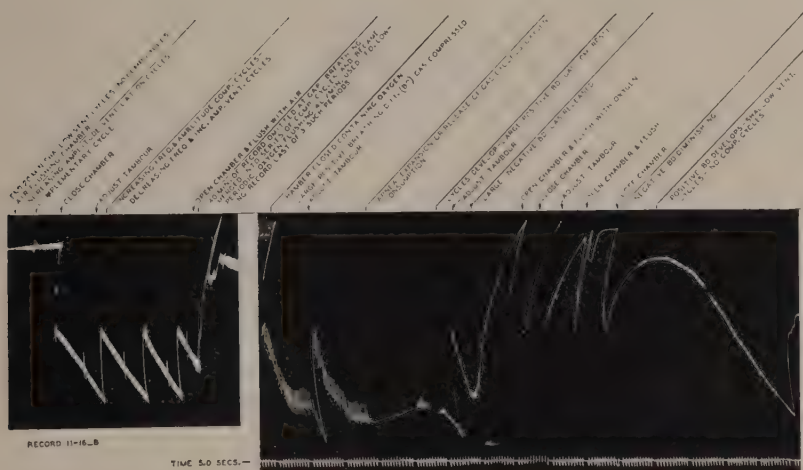
Fig. 9   Record from closed respiration chamber of periodic breathing in rat with an intra-tracheal cannula, to show intra-pulmonary alteration of breathing difference.

showed clearly a particularly important aspect of the question of intra-pulmonary gas retention, compression, and release; namely that these actions can occur during ventilation cycles through intra-pulmonary factors. This record is the last of three such abnormal breathing periods. There seems no alternative explanation to the magnitude and direction of variations in BD on this record except retention and compression by intra-pulmonary orifice constriction (bronchioles, alveolar openings) and breathing musculature contraction. The period of apnea almost two minutes long on the record is particularly interesting, because the normal RQ factors indicated in the first section of the recording are not reflected here. When

cycles begin again, however, the positive BD shown at the out-
set continues, which indicates that RQ factors had not altered
during the period of apnea, therefore respiratory areas must
not have been freely open to the atmosphere.

Interpretation of the volume changes recorded in these rat
studies required information about normal lung volumes. Re-

TABLE 5

Lung volume of rat

Observed: *Calculated:*[1]

EXP. NO.	WT. GM	TEMP. (ANALYSES) C. DEG.	RAROM. MM HG	N (SAT. H_2O) ML	(a) TRACT VOL., ML	(b) ALVEOLI VOL., ML
11–21	301 ♂	23.9	757.2	4.15 (end insp.)	5.7	4.12
11–23	339 ♂	25.1	755.2	3.87 (end exp.)	5.3	4.65

[1] Calculation: (a) let: N = 80.4% maximum of dry tract air
           Lung temp. = 40.0°C. (ref. A. G. Ware et al., '47)
           Vapor press. H_2O at 23.9 = 22.0 mm Hg
           Vapor press. H_2O at 25.1 = 23.7 mm Hg
           Vapor press. H_2O at 40.0 = 54.9 mm Hg

           then: total volume of gas in tract (saturated at 760.0 mm Hg)
               is found by substitution in formula, e.g.

$$\left(\frac{100N}{80.4} \right) \left(\frac{Bar. - 22.0}{760.0 - 54.9} \right) \left(\frac{273 + 40}{273 + T} \right)$$

      (b) let: Functional dead space (maximum) = 12.3% of functional
              residual air

          then: Alveolar air (air in contact with respiratory epithelium)
              is
              (11–21) [tract vol. (5.7) — tidal vol. (1.0)] × 87.7%
              (11–23) tract vol. (5.3) × 87.7%

sults and calculations for the total volume of the respiratory
tract and for that portion confined to respiratory areas are
given in table 5. So far as comparisons can be made from re-
sults of quite different methods, these results appear much
larger than those of Adelman and Hitchcock ('48). They found
a residual air volume of 0.87 ml average for rats of average
174.2 gm weight.

DISCUSSION

From the viewpoint of volume flow of air in terrestrial mammals the extreme differences in magnitude represented by horse (tidal volume 6000 ml — frequency 10/min.) and rat (tidal volume 1.5 ml — frequency 60/min.) are accompanied by important differences in the breathing mechanism. The polyphasic character of horse cycles and lack of complementary cycles, in contrast to diphasic cycles in rat and numerous complementary cycles, and the gradation of occurrence of complementary cycles in intermediate animals, these differences are clearly evident. Ellenberger and Scheunert ('10) recognized the dual aspect of horse expiration, and ascribed it entirely to a passive and active component of abdominal movements. The equally dual aspect of inspiration has been overlooked and makes the explanation of such a cycle more difficult. If the breathing movements of the body wall are recognized, however, to have two basic components, then explanation of the polyphasic cycle can be undertaken from a point of view which brings the horse and rat types into reasonable relationship. The two components of body wall movement to be emphasized are (1) they actively reflect the forces inducing air movement, and (2) they passively reflect changes in resistance to air movements. From this viewpoint, the complex movements during eupnea in the horse are related to concomitant alterations in intra-pulmonary resistance to air movement through correlated alteration in duct resistance. Such a rhythmical change is readily shown in nares, glottis, and trachea, but it is not so easily demonstrated for bronchioles (Ellis, '36) and other remote airways. Favoring this interpretation are (1) the intra-pleural pressure peak at the second phase of expiration, rather than the first in which the intra-tracheal and nasal pressures are highest, (2) the suppression of dual expiratory and inspiratory phases and peaking of the intra-tracheal pressure at the end of expiratory movement when bronchioles are dilated by epinephrine, (3)

the persistence of phases in intra-tracheal and nasal pressures
when the rapid breathing of hyperpnea has obscured them in
abdominal movements.

That rhythmical alterations in intra-pulmonary resistance
to flow is not inconsistent with limitations of smooth muscular
activity is suggested by the rhythmical alterations in intra-
caecal and rectal pressures. A pressure peak occurs at the end
of inspiration. This pressure peak must result from independ-
ent contraction of the intestinal wall, for the intra-peritoneal
pressure is undergoing a depression during this period which
is consistent with the inspiratory movements of the body wall.
Possible importance of this can be suggested. Movement of
the diaphragm in inspiration can occur only if (1) abdominal
musculature expands or releases tonus of the body wall or (2)
intestinal gases are compressed. Large volumes of intestinal
gases are present in herbivorous animals in comparison with
carnivorous animals. If this gas expands, in response to rela-
tive movement of abdomen and diaphragm, the effectiveness
of inspiratory movements is reduced. The active constriction
of the intestine at this point will offset the intestinal gas and
apply the entire abdominal movement toward alteration of
lung volume, or pressure. If intestinal musculature is thus
rhythmically active during each cycle, lung musculature can
similarly act to alter pulmonary resistance, volume, and pres-
sure during each cycle. It is of interest to find that Wislocki
('29) described a remarkable development of bronchiolar mus-
culature in the porpoise and specific bronchiolar sphincters in
the whale as adaptations to the peculiar breathing patterns
and pressure relations of aquatic mode of life in mammals.

Explanations of the observed factors in horse breathing, in
so far as diffusion regulation is concerned, may be summarized
in the following manner: Pressures appreciably above atmos-
pheric may be produced in alveoli during each breathing cycle
by (1) contraction of airway musculature to increase resist-
ance, (2) contraction of intrinsic air chamber musculature to
compress, and (3) contraction of body wall musculature. The

only available suggestion of the quantitative value of such a pressure comes from the observations on the horse which was restrained lying on its side (fig. 4, 1-27-A). In this position the weight distribution of abdominal viscera was greatly altered from normal. Expiration began with a rapid, large blast of air which was apparently the passive effect of the new weight distribution and not under control of expiratory muscles. A compensatory factor appeared in the form of a marked constriction of the glottis, which greatly reduced the outflow of air and held it at a relatively constant level until the end of the expiratory movement. Then the glottis released and another blast of air occurred. An intra-tracheal pressure of 18 mm Hg was typically maintained during the period of glottal restriction, and this must, of course, have been reflected at the respiratory surfaces. This might be a compensating mechanism regulating a pressure which normally develops and is regulated primarily by the intra-pulmonary factors previously outlined — if so, the pressure measurement indirectly evaluates the level of normal regulation.

The inferences concerning intra-pulmonary activities in horse breathing may now be applied to the rat. The short, one-second interval for a ventilation cycle in the rat would not permit smooth muscle of the lung to act as suggested in the horse. However, the 5-second interval occupied by a complementary cycle would do so. It is interesting that the complementary cycle period of 5 seconds is also the time occupied by a single breathing cycle in the horse. If an average level of diffusion pressure can be regulated in terrestrial mammals, then the complementary cycles in rapid breathers, like the rat, becomes meaningful from the viewpoint of diffusion regulation.

The results in this report show that some appreciable pressure does result intra-alveolarly at least during the complementary cycle of the rat. It is possible to make a first approximation of this pressure from the data presented, as follows:

1. Volume of alveolar gas at beginning of expiration: 8.59 ml

    (ref. 10–31) inspired: $4.7 \times \dfrac{762.1 - 28.1}{760.0 - 54.9} \times \dfrac{273 + 40}{273 + 28} = 5.09$ ml

    $5.09 \times 87.7\% = $ 4.47 ml added to alveolar
                     + 4.12 ml alveolar (ref. 11–23)
                     8.59 ml

2. Volume of intestinal gas (estimated from x-ray): 1.0 ml

3. Total volume of gas (40°C., saturated, 760 mm Hg.) subject to compression at
    beginning of complementary cycle: 9.59 ml

              8.59 alveolar gas
              1.00 intestinal gas
              9.59 ml

4. Average reduction in volume during cycle: 0.54 ml
    (Ref. 9–14, 10–4)

              $\dfrac{0.54 \times 761.1}{760.0}$

5. Intra-alveolar pressure at end of complementary cycle: 805.0 mm Hg
    (Boyle's Law) $PV = P'V'$ and $P' = \dfrac{PV}{V'}$

    V = 9.59 ml $P' = \dfrac{760 \times 9.59}{9.05} = 805.0$ mm
    V' = 9.59 − 0.54 = 9.05 ml
    P = 760.0 mm Hg

6. Cycle pressure: 45.0 mm Hg

              805.0 intra-alveolar pressure
          − 760.0 barometric pressure
              45.0

There are possible variables not included in such a calculation, and it must be regarded with suitable reservations. The assumption that RQ factors remain uniform during a cycle, for example, may be in error. A pool of reduced venous blood may be suddenly brought through the pulmonary circulation by this cycle, or restricted lung areas may be opened (Fowler, '49), and a temporary reduction of the oxygen may occur at an accelerated rate, without corresponding carbon-dioxide output. It does not appear, however, that this could account for more than possibly one-half of the observed volume reduction. The

greater magnitude and uniformity of complementary cycles in oxygen as compared with air might also seem accountable in terms of oxygen consumption. But the cycles in oxygen are also less frequent. A more likely explanation, therefore, involves the cushioning effect of nitrogen. In an oxygen atmosphere the consumption of oxygen would exert a smaller effect on total pressure in an alveolus, and possibly a more uniform effect among alveoli. The result is as though oxygen concentration in alveolar fluid, rather than oxygen tension, were integrated with total alveolar gas pressure.

That intra-pulmonary gas is uniformly dependent on ambient atmosphere, whether in composition (Fowler, '49), volume (Verzar, '46), or pressure (Einthoven, 1892; Keith, '09) has occasionally been questioned. Doubt has arisen because intra-pulmonary histology suggests a potentially independent regulator (Macklin, '29). Fowler gives evidence for uneven pulmonary ventilation in man, and Verzar gives evidence for volume regulation in rabbit and man. No physiological evidence for pressure regulation has been found heretofore. Otis ('48) attempted to evaluate intra-alveolar pressure in man. He assumed that with sudden stop of expiratory flow the pressure at the mouth equals that in alveoli. Measurements thus taken indicated a pressure of only 0.0035 cm H_2O per milliliter of air flow per second.

One physical factor which makes the suggestion of an average intra-pulmonary pressure of the order of 20.0 mm Hg in mammals difficult to accept is interference with capillary circulation. Pulmonary capillary pressure determined from indirect measurements in man, for example, is about 10.0 mm Hg (Hellems et al., '49). Thus 20.0 mm Hg pressure against the capillary could occlude the flow. It is conceivable, however, that the intrinsic elastic and muscle fibers of the walls of air sacs and pillars between alveoli, and the character of the alveolar membrane itself, may make the pressure of the gas chambers partially independent of the wall of the capillary as well as of the contraction of extrinsic breathing muscles. The functional inaccessibility of the normal pulmonary unit to a

direct quantitative study has resulted in total lack of direct information from which positive conclusions can be drawn. It is obvious, however, that it is at the level of intra-pulmonary diffusion factors that terrestrial mammals have the most common magnitude of physical dimensions, rather than in the gross volume flow factors of external breathing exchange. For example, an excellent series of measurements of alveolar dimensions recently made for 10 species of mammals by Hartroft and Macklin ('47), give striking evidence of the disparity between these functional levels and emphasize the common order of magnitudes involved at the pulmonary unit level. Their animals ranged in size from mouse to human. Alveolar dimensions ranged in size in the following order; mouse, rat, baboon, dog, goat, guinea pig, monkey, rabbit, cat, and human. Mean diameters in microns of alveolar mouths, for example, varied as follows: rat — 46.96, guinea pig — 67.38, rabbit — 69.63, cat — 98.68, dog — 57.17. Other dimensions were similarly related. In passages of this size the physical characteristics of gas movements become difficult to evaluate, as has previously been shown for the tracheolar system of insects (McCutcheon, '40a; Watts, '42). Physical regulation and buffering comparable to the nicely described chemical regulation and buffering of such substances as CO_2 appear to operate, however, and development of direct means to study them should be rewarding.

The significance of an independent, positive intra-pulmonary pressure is easily recognized. It relates to at least two important functions: (1) diffusion gradients in gaseous exchange, and (2) filtration pressures in capillary fluid exchange. Pertaining to the first function, a 5.0 mm Hg increase in the partial pressure of O_2 increases to 63% the saturation of a solution of rat hemoglobin which is otherwise one-half saturated (McCutcheon, '40b). Pertaining to the second function, the filtration gradient through which fluid is osmotically retained within pulmonary capillaries must lie between a capillary filtration pressure of about 10.0 mm Hg and colloidal osmotic pressure of about 25.0 mm Hg. A positive intra-alveolar pressure could

help maintain integrity of the intra-alveolar gas phase against fluid encroachment. Such encroachment (edema) is readily produced in rats by epinehprine and has been a subject of considerable study (Koenig and Koenig, '49). The problem of abnormally altered intra-pulmonary pressures (e.g. emphysema) might also be mentioned as related to the hypothesis of a regulated, average intra-alveolar pressure.

Certain new terminology in relation to breathing characteristics of mammals has been introduced to consider the results of these studies. These are (1) *breathing difference* (BD) and (2) *breathing coefficient* (BC), which emphasizes physical aspects of breathing in distinction from the chemical aspects involved in respiratory quotient (RQ). It is further proposed to designate the complementary cycles of the rat, and similar cycles in other animals if evidence of compression is obtained, as *compressatory cycles;* and to designate the total pressure of gases at the respiratory epithelium of air breathing animals as *pneumobaric pressure.* The latter term seems desirable, recognizing the great importance in respiratory exchange which such pressures can have, since it has been shown that in grasshoppers (McCutcheon, '40a), in turtles (McCutcheon, '43b) and now in rats the respiratory diffusion pressures are functionally somewhat independent from the atmosphere. The term will serve to emphasize pressure relations at respiratory membranes irrespective of the type of respiratory unit involved: whether alveolar, as in rat; pulmonary, as in frog; or tracheolar, as in grasshopper.

SUMMARY

Detailed study of breathing is presented for two terrestrial mammals of extreme size difference, horse and rat. Important differences in the two breathing processes are described, which are related to the divergent magnitude of factors in gross volume flow in and out of the respiratory tract. These are as follows:

1. Normal or resting cycles (eupneic) in the horse, moving about 6000 ml of air, occupy about 5.0 seconds. They are polyphasic, with dual expiratory and inspiratory phases. No complementary cycles occur.

2. Eupneic cycles in the rat, moving about 1.5 ml of air, occupy about 1.0 second. They are diphasic with a single expiratory and inspiratory phase. Complementary cycles occur in a rhythmical sequence at the rate of about 23.0 an hour.

The observed correlation of certain pressure peaks involved in horse breathing, particularly in upper respiratory passages and lower digestive passages as compared with those in thoracic and abdominal cavities, and their relation to breathing movements of body wall led to the suggestion of a significant intra-pulmonary pressure produced during each cycle. Support of this was inferred from the effect of inhaled epinephrine, which caused a diphasic type of cycle, and from the effect of altered abdominal weight distribution when the animal lay on its side, which resulted in glottal closure to create a pressure of 18 mm Hg in the trachea with each cycle.

The complementary breathing cycles, which are shown to occur in rat, guinea pig, rabbit, dog, and man, are described and analyzed in detail for the rat. Their characteristics appear also to indicate that a significant intra-alveolar pressure is involved in breathing regulation. These characteristics are as follows:

1. Complementary cycles in the rat move about 4.5 ml of air.

2. They occupy about 5.0 seconds (the duration of eupneic cycles in horses) and they occur at intervals of 2 to 3 minutes.

3. They consist of three phases; a rapid inspiration and expiration, and a slow compression.

4. A portion (0.3 to 0.7 ml) of the inspired volume (4.5 ml) is usually retained by intra-pulmonary restriction.

5. The retained volume and residual volume at atmospheric pressure are reduced during the compression phase to a volume equal to that of the residual volume.

The relative magnitudes of breathing (physical) and respiratory (chemical) factors involved in the complementary

cycles of rats indicate that the reduction in volume which occurs during the compression phase results in development of a significant intra-alveolar pressure. Alveolar volume determined for the rats was about 4.0 ml. This value, together with others which were estimated (intestinal gas volume, lung temperature, dead space), was used to calculate an approximate order of magnitude for the intra-alveolar pressure produced during the cycles. A pressure of about 20 to 40 mm Hg is indicated. This must be regarded only as tentative evaluation because of limited data and knowledge of variables.

It is concluded that the existence of comparable magnitudes of factors involved in diffusion regulation at the respiratory membranes can help to explain the described differences in gross breathing patterns among animals, and that independently regulated intra-alveolar pressure is one these factors. The term *pneumobaric* is proposed to describe this pressure.

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SOME FURTHER INFRARED ABSORPTION STUDIES ON THE PROTEINS OF MUSCLE [1]

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FIVE FIGURES

INTRODUCTION

In an earlier paper (Morales and Cecchini, '50) there were reported a number of observations on the infrared absorption of substances involved in the muscle system. The present note extends certain of these observations and introduces some new ones. The various points will be considered separately below.

EXPERIMENTAL

The actin employed in these investigations was prepared by the method of Feuer et al. ('48); myosin, by the method of Spicer and Gergely ('50); and fibrinogen, by the method of Laki (in preparation). A few measurements were made on commercial samples of insulin (Lilly) and serum albumin (Armour). Actomyosin was prepared by mixing F-actin with myosin in the optimal combining proportions (Spicer and Gergely, '50). G-actin was polymerized to F-actin by bringing the K^+ concentration to 0.1 M (Straub, '43). Spectroscopic technique and instrumentation were as previously described (Morales and Cecchini, '50).

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[1] The opinions or assertions contained herein are the private ones of the writers, and are not to be construed as official or reflecting the views of the Navy Department or naval service at large.

[2] Public Health Service Special Research Fellows of the Experimental Biology and Medicine Institute.

2100–2200 cm^{-1} bands. Bands at 2100–2200 cm^{-1}, to our knowledge not previously reported in muscle proteins,[3] were definitely found in myosin, actin, and actomyosin (fig. 1). This absorption had been previously noted by us in actomyosin, and in muscle tissue itself. We were not able to detect it in fibrinogen. No assignment as a fundamental (e.g., Randall et al., '49) or as an overtone is obvious.
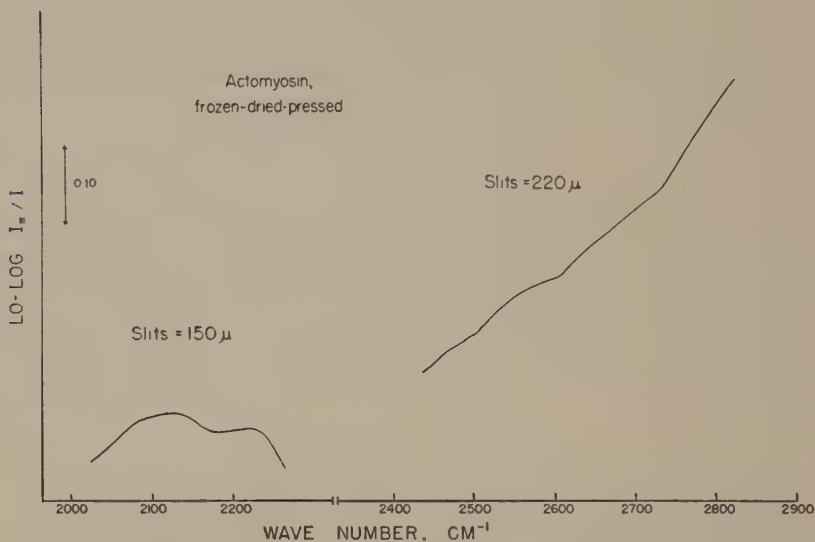
Fig. 1 Portion of the high-frequency spectrum of actomyosin. The right-hand portion of the spectrum, showing the faint bands believed to be S-H stretching absorptions, was measured on a specimen so thick that the C-H and N-H transmittances were virtually zero.

Sulfhydryl bands. It has already been shown by us that absorptions due to S-H stretching vibrations are absent under conditions in which C-H and N-H absorptions are well resolved. By using very thick specimens (in which N-H and C-H transmittances are of baseline strength), however, we have located faint absorptions in the neighborhood, 2600–2700 cm^{-1}

[3] Bands at this frequency, however, were reported by Stair and Coblentz ('35) in egg albumin and in egg membrane. We are grateful to the N.I.H. referee board for calling the reference to our attention.

(figs. 1 and 2). These absorptions, which we believe to be due to sulfhydryl, have been found in actomyosin, myosin, and insulin, but not in actin or fibrinogen; this is in accord with corresponding chemical information. The mere finding of these weak bands does not, of course, dispose of the S-H problem
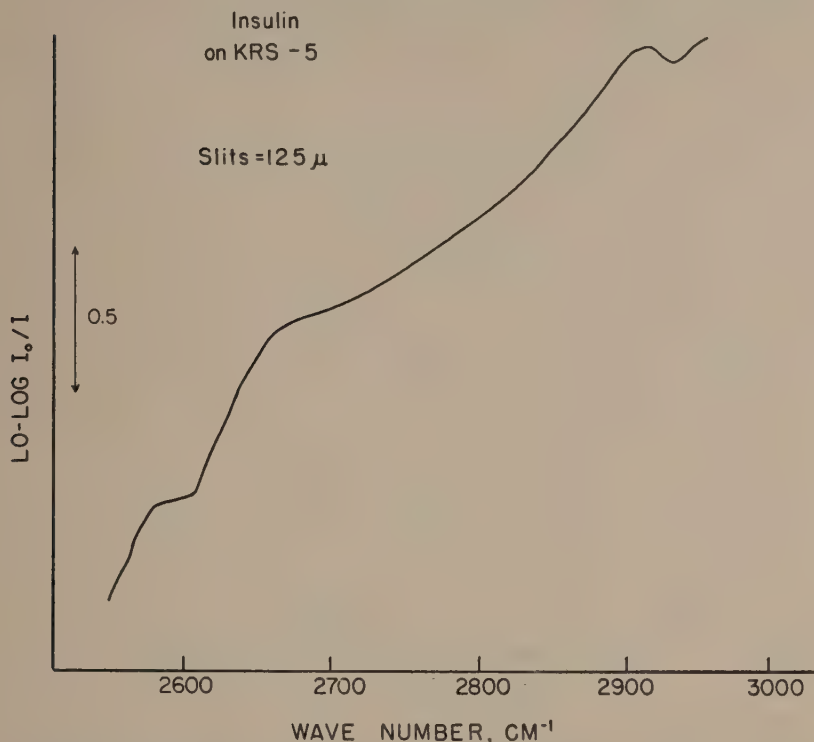
Fig. 2 High-frequency spectrum of a thick specimen of insulin, showing 2600–2700 cm⁻¹ bands believed to be due to sulfhydryl groups.

discussed in an earlier paper (Morales and Cecchini, '50). It is probable that the question as to what per cent of the sulfur groups is in the S-H form can only be settled by making absolute intensity measurements which are at present beyond our instrumental facilities.

The 930 cm⁻¹ band. It has been shown earlier that adenosine diphosphate (ADP), deposited from a solution at pH 7,

actomyosin, and actomyosin-ADP mixtures have absorptions
near $930\,cm^{-1}$, and it has been suggested that the $930\,cm^{-1}$
band in the protein might arise from tightly bound ADP. As
was indicated at that time, the possibility that the band might
arise partly from a vibration in the protein itself, and that
the two frequencies coincided accidentally, could not be ruled
out. The conclusion that ADP (or its deaminated form) is
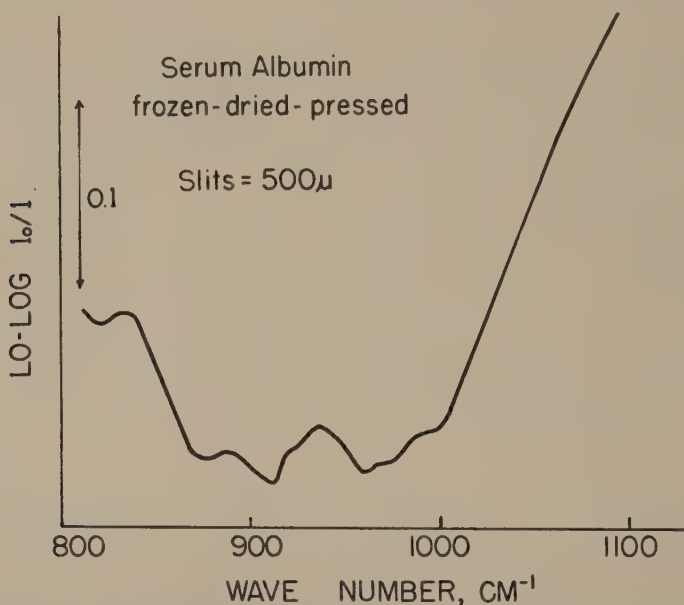
Fig. 3 Portion of serum albumin spectrum to show existence of a $930\,cm^{-1}$
absorption in the absence of nucleotide.

bound to (the actin portion of) actomyosin is probably cor-
rect, as shown independently and by different methods by
Laki et al. ('50) and by Straub and Feuer ('50). However,
the present infrared work has shown that there is indeed an
accidental coincidence of frequencies: Serum albumin (fig. 3)
and fibrinogen (fig. 4), neither of which contain orthophos-
phate or nucleotides, show a $930\,cm^{-1}$ band, and actin from
which some 75% of the nucleotide has been removed (by
isoelectric precipitation and dialysis; see Laki et al., '50)

still shows substantial absorption in this region. Under these circumstances it is clear that the interpretation of changes in this band must proceed with caution (see below).
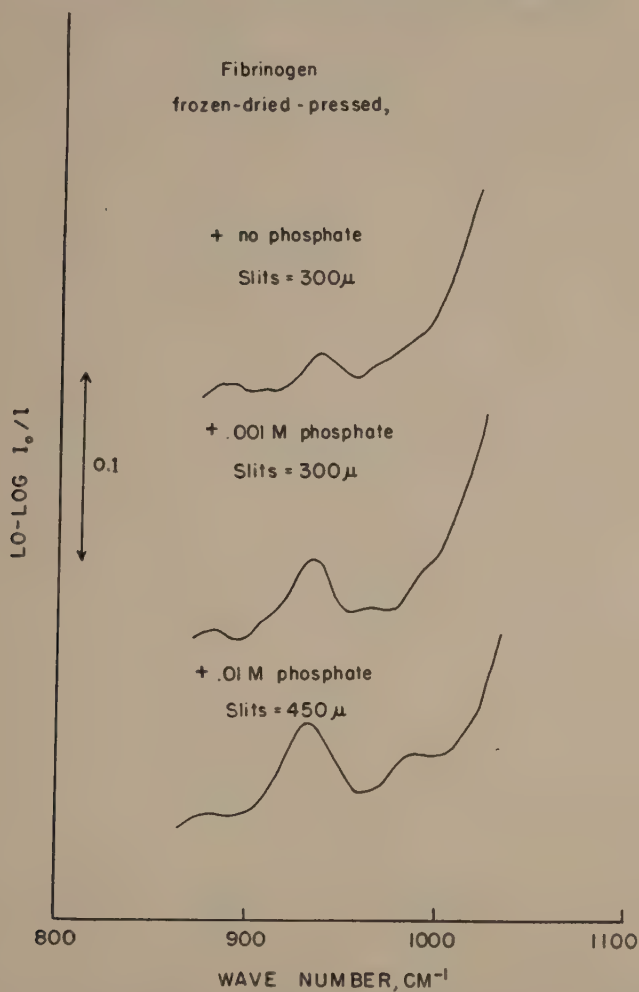
Fig. 4   Spectra of mixtures of varying amounts of inorganic (orthophosphate) with fibrinogen to show growth of 983 cm⁻¹ band parallel to increase in phosphate content. There appears to be likewise a growth of the 930 cm⁻¹ band; this last is unexplained. In this comparison there were technically unavoidable differences in film thickness, hence in slit width. It should be noted, however, that the 983 cm⁻¹ band appears under the least favorable conditions for resolution (widest slits) so that its appearance must be regarded as real.

The 983 cm⁻¹ band. Further evidence has been obtained indicating that the 983 cm^{-1} band in orthophosphate-protein mixtures arises from bound phosphate. The strength of this band in different preparations of the same protein has been found to parallel the phosphate content (e.g., fig. 4). A further indication of the interaction is the fact that with roughly equal orthophosphate-protein (weight) ratios this band has been found to be stronger in mixtures with myosin or actin than in mixtures with fibrinogen.

Actomyosin formation. Except for differences discussed above — those due to variable phosphate content and to sulfhydryl — the spectra of actin, myosin, and actomyosin [4] were found to be very similar. This similarity implies that there is no drastic change in the covalent bond structure as a result of actomyosin formation. In particular, the S-H absorption in actomyosin is about as strong as in myosin, contrary to what would have been expected if the S-H groups were transferred to S-S groups in the course of the combination of actin with myosin. This result does not necessarily contradict the experimental finding (Bailey and Perry, '48) that the S-H groups are essential for the combination, since conceivably such groups could participate in an interaction other than oxidation.

Changes with polymerization of actin. Laki et al. ('50) and Straub and Feuer ('50) have shown that unpolymerized G-actin contains bound ATP. Upon addition of KCl the system polymerizes to F-actin, and there is a concomitant increase in inorganic phosphate as well as in ADP (and possibly in its deamination product). These findings suggested that the polymerization reaction might have spectroscopic counterparts in the 930–983 cm^{-1} regions. Such has been found to be the case. In passing from G-actin to F-actin the 930 cm^{-1} band increased relative to the 983 cm^{-1} band (fig. 5). Although the empirical fact is clear enough, any

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[4] Furthermore, the spectrum of actomyosin prepared from actin and myosin, as in this paper, was very similar to the spectrum of actomyosin extracted as such (Morales and Cecchini, '50).

interpretation can only be tentative. The increase of the
930 cm^{-1} band might be considered to be due to changes in
the protein structure on polymerization. Owing, however, to
the fact that ADP has an absorption at this frequency the
following two interpretations have also to be considered. A
relative increase in the 930 cm^{-1} band would be consistent
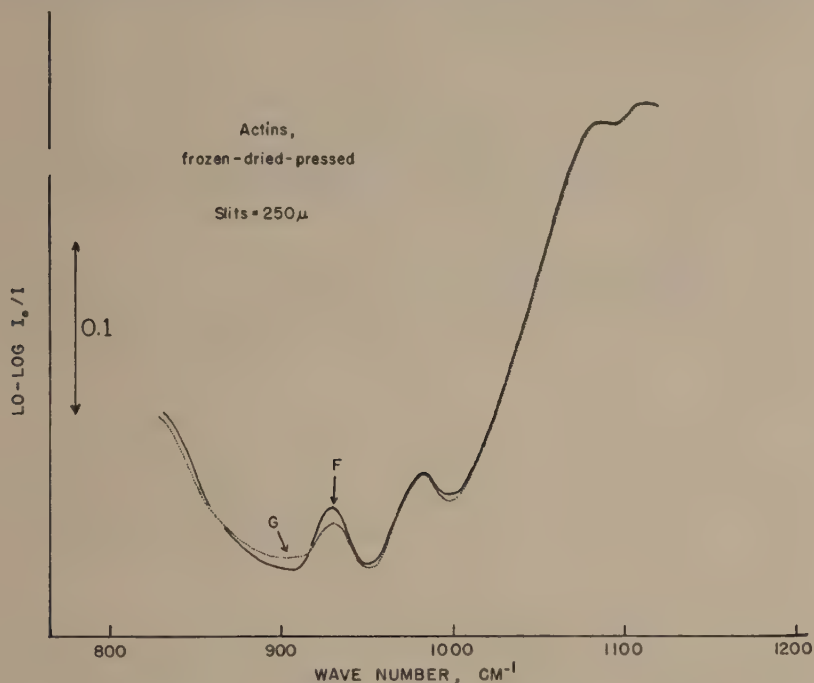
Fig. 5   Comparison of the spectrum of G-actin with F-actin. The specimens
differ only in KCl content.

with an increase in orthophosphate and nucleotide diphos-
phate provided we assume that the ADP absorption is stronger
than the orthophosphate absorption (which seems to be the
case); however, this suggestion implies that there should be
a parallel decrease in the 911 cm^{-1} absorption due to ATP
(Morales and Cecchini, '50), and this decrease, although
suggested by figure 5, is not localized enough to ascribe
unequivocally to ATP. Another interpretation would be to

assume that the $930\,cm^{-1}$ band remains constant (it being supposed that it arises almost exclusively from protein vibrations) and that the $983\,cm^{-1}$ band decreases. This idea, however, would be in conflict with the analytical fact that there is more orthophosphate in the F-system than in the G-. Tentatively, therefore, the first of the hypotheses involving ADP seems to be more likely.

SUMMARY

1. Previously unreported absorption bands were found in the spectra of myosin, actin, and actomyosin, at frequencies in the region $2100\text{--}2200\,cm^{-1}$. These bands are strong in muscle tissue.

2. Weak bands, believed to be sulfhydryl, were found in the spectra of actomyosin, myosin, and insulin (but not in actin or fibrinogen), at frequencies in the regions, $2600\text{--}2700\,cm^{-1}$.

3. The protein structure itself (in actin, serum albumin, fibrinogen, and perhaps other proteins) was found to give rise to an absorption at $930\,cm^{-1}$, a frequency coincident with one of the main absorption frequencies of adenosine diphosphate. This complicates, but does not necessarily exclude, the spectroscopic study of the nucleotide band.

4. The spectra of actin and myosin were found to be quite similar (the few dissimilarities appeared to be interpretable), and no gross changes occurred when the two proteins combined to form actomyosin. In particular, the bands of myosin believed to be S-H bands appeared just as strong in actomyosin.

5. Upon polymerization of G-actin to F-actin, the $930\,cm^{-1}$ absorption band increased relative to the $983\,cm^{-1}$ (bound orthophosphate) band. A plausible interpretation of this phenomenon can be given in terms of the fact that G-actin contains tightly bound ATP, and that in the course of polymerization this ATP is dephosphorylated to ADP.

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THE ACTION OF TEMPERATURE, CYANIDE AND AZIDE ON THE CARDIAC AND RESPIRATORY RHYTHMS OF GAMMARUS CONFERVICOLUS (STIMPSON)

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THREE FIGURES

INTRODUCTION

The heart beat and respiratory rhythm of the amphipod *Gammarus confervicolus* (Stimpson) are readily observed under a binocular microscope ($24 \times$ magnification). The heart is a dorsal pulsating tube lying between the transparent exoskeleton and the gut. The respiratory activity consists of the rhythmic movements of the pleopods which wash water over the gills. A close correlation between the two rhythms is evident. The object of this set of experiments was to ascertain whether the control of these mechanisms was due to the same or different sets of enzyme reactions. To test this point, the effects of temperature and certain respiratory poisons on these rhythms were observed. If the correlation between these rhythms were destroyed by the use of these agents, different controlling mechanisms would be indicated. However, if the correlation did not break down, it might be concluded that these mechanisms are controlled by certain similar enzymes.

Several mathematical formulae have been proposed to express the relationship between temperature and the rate of biological processes (Bělehrádek, '35). Their relative merits need not concern us since they are used here only to compare the effects of temperature on rates of activity of two different processes in the same animal.

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Cyanides and azides have been shown by Keilin ('36) and others to inhibit cellular respiration. The inhibitory effect of hydrocyanic acid on the cytochrome oxidase system of respiratory enzymes is believed to be due to its combination with the ferric compound. Keilin ('36), noting the similarity of effects of sodium azide and sodium cyanide, suggested that these two inhibitors may act at the same point in the catalytic system of cellular oxidation. However, several workers (Armstrong and Fisher, '40; Spiegelman and Moog, '45; Stannard, '39) have shown basic differences in the action of these agents. From the results of these investigations it seems reasonably certain that cyanides and azides act on different enzyme systems. A comparison of their actions *may* show whether the same or different enzyme systems are involved in the control of the cardiac and respiratory rhythms of *G. confervicolus*.

MATERIALS AND METHODS

The amphipods, *Gammarus confervicolus* (Stimpson), were caught in a small stream running through the Musqueam Indian Reservation on south-west Point Grey, Vancouver, B. C. The first series of experiments was carried out in January and the second series in July. The temperature of the stream in January was approximately 6°C., while in July it was about 14°C. The most satisfactory method for retaining the animals in the laboratory was to keep them in an enamel bucket along with water and debris from the stream. The bucket was kept in a bath of running tap water (approximately the same temperature as the stream at both seasons) and compressed air was passed through the water in the bucket. The animals, retained in this manner, appeared to be normal and healthy for a period of several months.

The apparatus used for the examination of temperature characteristics was set up as follows: a three-liter flask used as a reservoir was placed in a thermostatically controlled water bath. The reservoir was fitted with a siphon to maintain a constant level of water. Another siphon led to a piece of glass tubing containing the animal under observation. A small amount of glass wool was inserted in the tube on either side of the animal to keep it in place. Three thermometers were used to check the temperature throughout the system, one in the reservoir, one in the thermostatic water bath, and one just before the water entered the observation tube. This last thermometer was graduated to 0.01°C. and this is the temperature reported here. Constant illumination was obtained from a Spencer microscope lamp provided with a daylight filter.

At each temperature the duration for 20 respiratory and 20 cardiac movements were taken alternately and an average of each 20 such measurements for each particular rhythm was taken as a "reading." A stopwatch was used for timing. In one set of temperature experiments the temperature was raised 1°C. between each such set of "readings" and the animals were acclimatized to each increase in temperature for approximately 15 minutes. In another set, the temperature was varied at random.

The experiments using the sodium cyanide or sodium azide were carried out at constant temperature in essentially the same manner. The sodium cyanide and sodium azide solutions were freshly prepared each day. Since pH has been shown to affect the action of these compounds on tissues (Armstrong and Fisher, '40), this factor was rigidly controlled. The pH was adjusted by means of a Beckman meter, using sodium hydroxide or hydrochloric acid as required. For the control experiments of pH, tap water was adjusted to the proper pH in the same manner. A solution of carbonic acid was also used to test the effects of pH.

RESULTS

1. *Correlation of rhythms under normal conditions*

It was essential to know first of all just how constant the rhythms were for a single animal at a constant temperature. To study this variability 20 trials of each animal were taken at 6°C. (approximate environmental temperature in January). Another set of data was obtained in July at 14°C. These

TABLE 1

Cardiac and respiratory rhythm of Gammarus. The rate is the average of 20 determinations of the time required for 20 movements — cardiac and respiratory time intervals being recorded alternately

SEX	LENGTH	HEART		RESPIRATION	
		Rate	Standard error	Rate	Standard error
	mm	*sec.*	*sec.*	*sec.*	*sec.*
(a) Results in January at 6°C.					
M	9.5	10.88	0.03	9.25	0.05
F	6.4	10.98	0.05	9.31	0.08
F	5.1	11.02	0.02	9.29	0.03
M	12.0	10.99	0.04	9.16	0.04
F	5.3	10.90	0.06	9.27	0.02
M	8.5	11.10	0.03	9.28	0.05
(b) Results in July at 14°C.					
M	11.5	7.43	0.04	6.74	0.06
F	6.4	7.43	0.03	6.68	0.06
M	9.8	7.38	0.03	6.82	0.09
M	15.2	7.52	0.06	6.73	0.05

data were analyzed statistically (Snedecor, '46) and are presented in table 1.

From the results in table 1, it is evident that there is almost no variation in the rhythms under normal conditions, since the original readings were taken to the nearest one-tenth of a second and the standard error is considerably less than this.

MacArthur and Baillie ('29) found that male *Daphnia* had higher heart-beat rates than females. This condition might also be expected in *Gammarus* but is not apparent in the

analysis presented in table 1. The sample is composed of both males and females of varying sizes. The standard error of the mean for each animal is small. The fiducial limits, at the 95% level, for combined readings of heart beat were found to be $\pm$ 0.118 and for respiration $\pm$ 0.158 seconds, thus indicating little variability. In addition, the data presented in figures 1 to 3 do not show inconsistencies which might be due to different rates in the two sexes and suggest further that this factor is unimportant in the present experiments.

2. *Correlation between rhythms in relation to temperature*

Graphic treatment of the temperature data, according to the methods of Crozier ('22–'31), shows a very close similarity in temperature characteristics of heart and respiratory rhythms (fig. 1). The graphs are based on average figures for 6 animals. The results obtained in July parallel those obtained in January. The correlation coefficient (r) between the rate of cardiac and respiratory rhythms with regard to temperature was calculated and found to have a value of $+$ 0.97. This indicates an extremely close correlation and leads to the conclusion that both rhythms are affected in the same manner by temperature. It should, however, be pointed out here that both rhythms became very irregular at 4° to 5°C. below the lethal temperature and such data are not considered in this analysis. Essentially the same results were obtained when the temerature was varied at random. The correlation was still almost perfect ($+$ 0.92).

3. *Correlation between rhythms using cyanide and azide*

To select an appropriate concentration of cyanide or azide for use in these tests, observations were made on the effects of different dilutions. In one series the pH was maintained at 7.0 and concentration varied from M/500 to M/2,000. In another series with pH = pK the concentration was varied from M/1,000 to M/10,000. At pH = pK the acid is least

dissociated and should perhaps have a maximum effect since it would be most permeable in the undissociated form.

The effect of concentration (pH = pK) is shown in figure 2. The inhibition levels for the cyanide on both rhythms and for the azide on cardiac rhythm were about 50% to 65% with
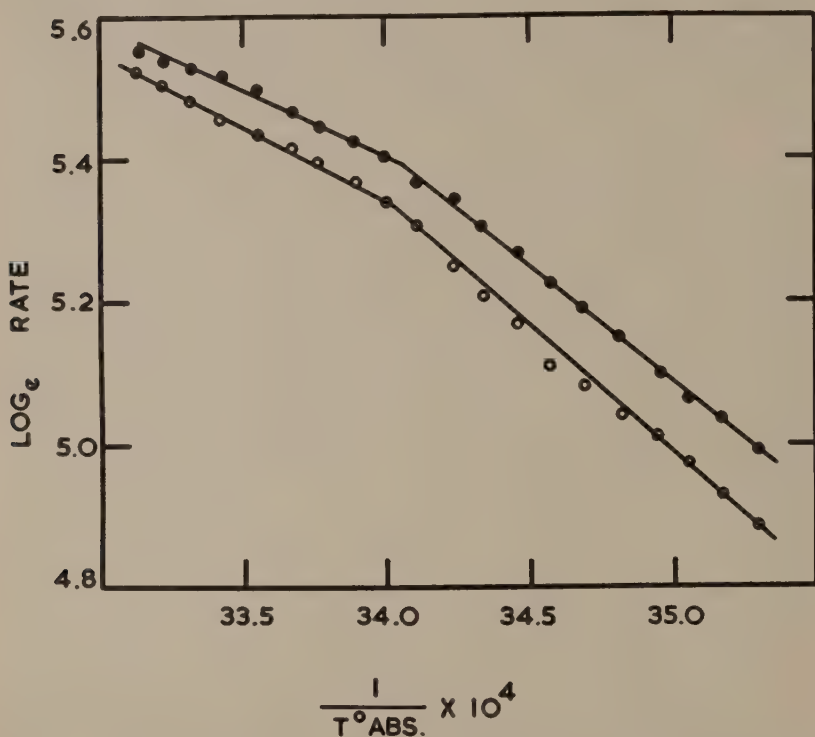
Fig. 1   Effect of temperature on cardiac and respiratory rhythm of *Gammarus*.

Graph showing log$_e$ rate (beats/min.) plotted against the reciprocal of the absolute temperature. Solid circles, respiratory rhythm; open circles, cardiac rhythm. The results shown are averages for 6 animals taken in January.

the M/1,000 solutions both at pH = pK as well as at higher and lower pH values. This concentration was arbitrarily chosen for the series of experiments that follow.

I. *Tap water, pH 6.0 (carbon dioxide)*. In this experiment (fig. 3A) there was at first a slight drop in the respiratory

rate which then increased to about 20% above normal. The heart, however, maintained its normal rhythm. This effect of an increase in the respiratory rate was almost duplicated by substitution of water adjusted to the same pH with HCl. This has been observed by other investigators (Scheer, '48).
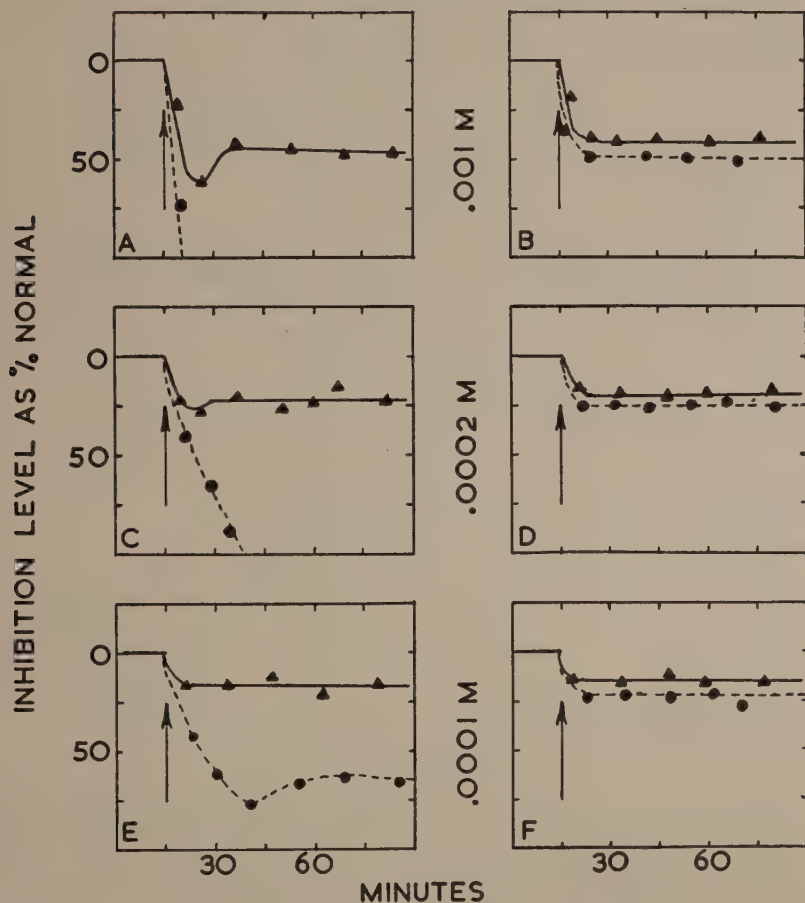
Fig. 2   Effect of different concentrations of cyanide (pH = pK = 9.2) and azide (pH = pK = 4.7) on the cardiac (solid lines) and respiratory (broken lines) rhythms of *Gammarus*.

Left, for azide; right, for cyanide; A-B, M/1,000; C-D, M/5,000; E-F, M/10,000; arrows, point of application of inhibitor. Temperature varied between 20°C. and 22°C. in different tests but was constant for any one test.
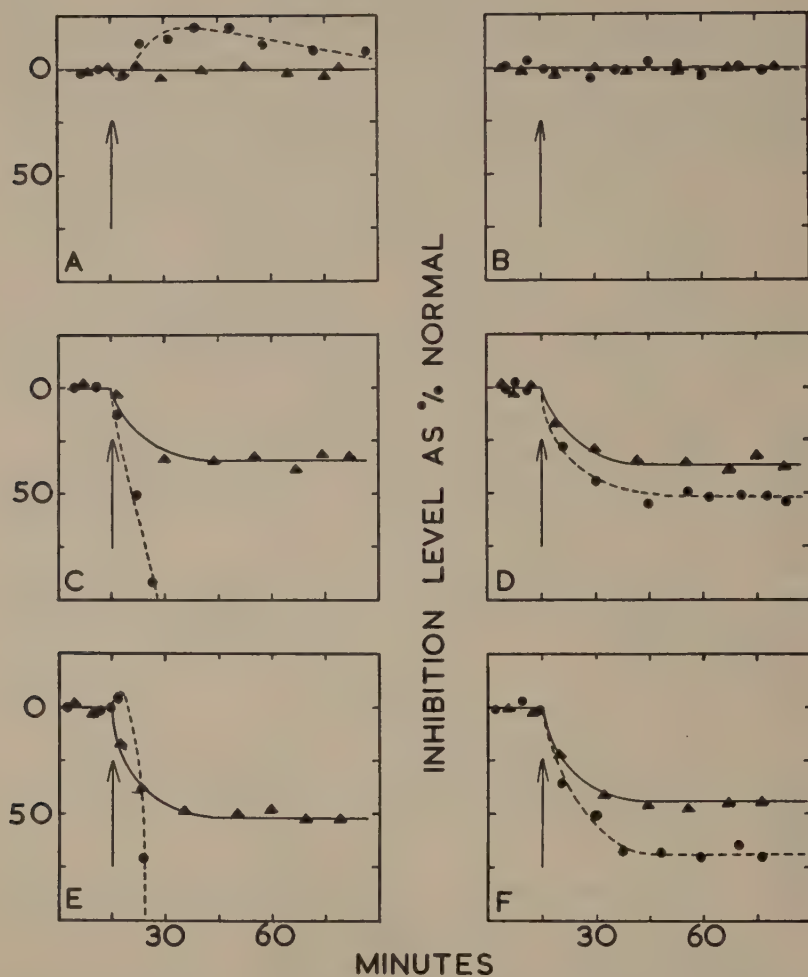
Fig. 3 The effect of acidity (upper graphs), cyanides (middle graphs) and azides (lower graphs) on cardiac (solid lines) and respiratory (broken lines) rhythm of *Gammarus*, based on values obtained in July. Arrows show point of application of reagent. Temperature varied between 20°C. and 22°C. in different tests but was constant for any one test.

       A.   Carbonic acid solution, pH 6.0 — 3 animals
       B.   Alkaline (NaOH) solution, pH 8.0 — 2 animals
       C.   NaCN M/1,000 pH 6.0 — 6 animals
       D.   NaCN M/1,000 pH 8.0 — 4 animals
       E.   NaN$_3$ M/1,000 pH 6.0 — 5 animals
       F.   NaN$_3$ M/1,000 pH 8.0 — 4 animals

II. *Tap water, pH 8.0 (sodium hydroxide)*. This solution did not alter the normal rhythms of either the respiratory or cardiac movements (fig. 3B).

III. *Sodium cyanide, pH 6.0*. The heart rate fell to an inhibition level of approximately 68%. This was very close to the level produced by a neutral (pH 7.0) solution of cyanide of the same concentration. The respiration, on the other hand, dropped off to zero within a very short time. No inhibition plateau appeared (fig. 3C).

IV. *Sodium cyanide, pH 8.0*. With this solution both the respiratory and cardiac rhythms reached a stable inhibition level, the heart at 63% and the respiratory movements at 48% of the normal (fig. 3D).

V. *Sodium azide, pH 6.0*. The acid azide appeared to stimulate the respiration when the animal was first immersed. However, this was only momentary and the rhythm was completely inhibited in a manner similar to the acid cyanide. The heart rate was inhibited to approximately 48% of the normal (fig. 3E).

VI. *Sodium azide, pH 8.0*. The basic azide inhibited the heart to a lesser extent than the acid azide. The inhibition level in this case was 56% as compared to 48% in the latter case. The respiratory rhythm was inhibited to about 32% of the normal rate (fig. 3F).

VII. *Cyanide (pH = 9.2) and azide (pH = 4.7) at pH = pK*. The time taken to reach an inhibition level is less when the pH is equal to the pK (figs. 2 and 3). Thus it appears that although the inhibition level is not appreciably changed in the case of the cyanide, the rate of inhibition is hastened. This also appeared to be the case with the azide, although here the pK is on the acid side and the respiratory rhythm was completely inhibited.

DISCUSSION

The results show that the rhythms behave in the same manner as the temperature is varied with a critical break in the Arrhenius plot for both rhythms at about 20°C. It is

interesting that results obtained in summer and winter were so similar. Barcroft ('40) has described seasonal variations in the effect of temperature on the heart of the frog and some other hibernating animals by plotting the frequency of the heart beat against the reciprocal of the absolute temperature (Arrhenius plot). The amphipods used here do not hibernate and this may account for the similar picture in both seasons.

Barcroft ('40) has studied the effects of temperature on both cardiac and respiratory rates of several species and has found that the pictures are very similar. For some animals, at least, there is a suggestion of a break (Arrhenius plot) of exactly the same nature for both rhythms. For the frog this seems to occur at about 20°C. This also appears to be the approximate temperature at which the break occurs in the experiments with *G. confervicolus*.

Stier and Wolf ('32) in their study of cardiac and respiratory rhythm of *Daphnia* found that there was a critical temperature (break) for gill movements but no such break for the cardiac rhythm. However, an examination of their data suggests that there may have been insufficient observations on heart beat near the critical temperature for respiration. A slightly different interpretation with more points might show these curves to be more nearly parallel.

The picture of cyanide and azide inhibition of the heart of *G. confervicolus* is similar to that obtained by Fisher and his co-workers (Armstrong and Fisher, '40; Fisher and Öhnell, '40) for embryonic fish hearts. In every case the heart of *Gammarus* was inhibited by the poisons, but, with M/1,000 solutions, never completely stopped. The respiratory rhythms, on the other hand, showed an entirely different picture and varied decidedly with the pH of the solution. In every case, the effect on the respiratory rhythm was more pronounced than the effect on the heart rate. In addition, acidic solutions of cyanide or azide bring about complete and rapid inhibition of the respiratory rhythm. From the differences in the effects of the solutions it would appear that there is some difference in the control of the rhythms.

Control experiments with water adjusted to different pH values demonstrated that the above effects were not due to pH alone. In contrast to the acidic poisons the acidic (H_2CO_3) control solution sharply increased the respiratory rate which reached a maximum and then slowed toward the normal. Similar results have been obtained with many other arthropods. Scheer ('48) states that this effect may be duplicated with hydrochloric acid. This was also found to be the case in the present experiment. The heart showed little response, if any, to pH alone. The water at pH 8.0 had little effect on either the cardiac or respiratory rhythms.

The acid poisons completely inhibited the respiratory motions of the pleopods but not the heart, and pH alone stimulates the respiratory rhythms. It would not seem to be a case of differential permeability since both actions depend upon active striated muscle within the body. Cyanides and azides inhibit activities by interfering with the action of certain oxidizing enzymes of cells (Keilin, '36; Fisher and Öhnell, '40). Since the cardiac rhythm of *Gammarus* is depressed but not completely inhibited it is suggested that the enzyme systems governing this rhythm contain both cyanide-azide sensitive and cyanide-azide insensitive fractions. The respiratory rhythm, on the other hand, is completely inhibited by these reagents and would seem to be entirely controlled by cyanide-azide sensitive enzymes. Thus, it appears that different controls (enzymes) are responsible for the maintenance of these rhythms.

Armstrong and Fisher ('40) find that the heart frequency inhibition is proportional to the concentrations of free hydrazoic acid in a solution of sodium azide. The fact that external pH does influence the degree of inhibition, and the fact that it is the free hydrazoic acid which is the active inhibitor, indicates that it must be only the azoimide which can enter the organism from the external solution. The results of these experiments are in line with the findings of this paper for the azide solutions. Fisher and his workers did not find that pH affected the cyanide action while our results show very striking

effects on the respiratory rhythms in particular. Acid cyanide solutions are similar in action to acid azide solutions and, in case of *Gammarus,* any theory formulated for the effect of azide must also apply to the effect of cyanide.

SUMMARY

1. A highly significant correlation has been shown to exist between the cardiac and respiratory rhythms in *Gammarus confervicolus.*

2. There is no essential difference in the effects of temperature on these rhythms.

3. Both respiratory and cardiac rhythms are sensitive to cyanide and azide under all conditions of pH tested (pH less than 7, pH greater than 7, and $pH = pK$).

4. Cyanide and azide under similar conditions of pH and concentration have similar effects on heart and respiratory rhythms.

5. The respiratory rhythm was completely sensitive to both inhibitors at appropriate concentrations and pH (concentration not less than 0.002 M and an acid pH).

6. The cardiac rhythms showed a cyanide and an azide insensitive fraction under all conditions of pH and concentration. This residual fraction is of the order of 50% of the normal rate.

7. It is concluded, therefore, that the enzyme systems for the control of cardiac and respiratory rhythms are, at least partially, different, although the enzymes may catalyze similar types of reactions as indicated by their response to temperature.

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COMMENTS
AND
COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

THE NON-SPECIFIC NARCOTIC ACTION OF DFP

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ONE FIGURE

The anti-cholinesterase action of DFP is well established, and the drug has been employed frequently in recent years in studies of nerve physiology; the concentrations employed in these studies, however, have been very much higher than those required to inhibit cholinesterase; thus Hawkins and Mendel ('47) state that a concentration of 1.10^{-6} M is adequate for this purpose whereas Rothenberg ('50) used a concentration of 0.02 M, i.e., 5,000 times as strong, in his study of the effects of DFP on nerve permeability, and Bullock et al. ('47) a concentration of 0.015 M in their studies of the optic nerve of the skate. DFP is highly lipoid-soluble, and

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may therefore be expected to show a non-specific narcotic action; if this expectation is confirmed by experiment it may well be that some of the actions attributed to a blocking of cholinesterase are, in effect, due to a less specific influence of the drug. Narcotics generally increase the permeability of natural membranes (for a review vide e.g. Davson and Danielli ['43] Chapter no. XVI); in certain cases, however, they more or less completely block permeability, e.g. the penetration of glycerol into the human erythrocyte. The cat
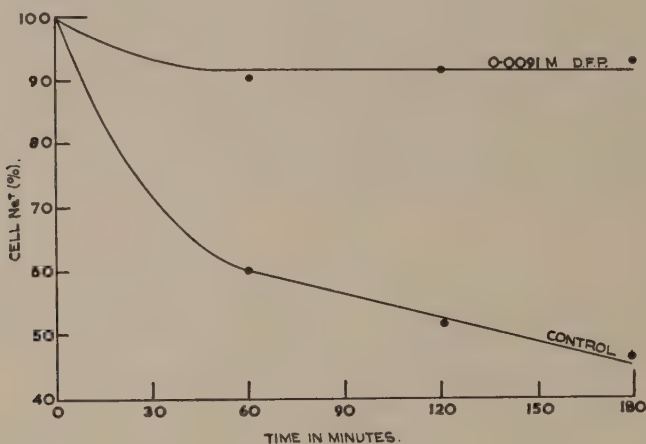
Fig. 1 Effect of DFP on escape of Na+ from cat erythrocytes into KCl solution.

Ordinates: Cell Na+, as percentage of original content.

Abscissae: Time in minutes.

Note that DFP inhibits the escape.

erythrocyte, suspended in isotonic KCl, exhibits both these characteristics (Davson, '40), the penetration of K^+ into the cell being accelerated by narcotics whilst the escape of Na+ from it may be blocked by an appropriate concentration.

Figure 1 shows the effect of a concentration of 0.0091 M DFP on the escape of Na+ from cat cells suspended in KCl solution. There is no doubt that the drug exhibited a narcotic action, similar to that of alcohols, urethanes, heavy

metals, soaps, etc., in a concentration less than that used in studies of nerve function.

It is not intended to enter into the controversy regarding the function of cholinesterase in nerve conduction, beyond drawing a legitimate conclusion from this experiment, namely that a cell or tissue treated with concentrations of DFP in the region of 0.01 M is definitely being submitted to a non-specific narcotic action.

In conclusion we may remark on the significance of the term ''DFP concentration;'' DFP is only slightly soluble in water, and is, moreover, decomposed by this solvent; consequently it is difficult to specify the actual concentration at any moment. The concentration in a given bathing medium for a tissue will also be critically determined by the relative amounts of tissue and bathing medium, since the lipids of the tissue will take up sufficient to affect the aqueous concentration. It is therefore necessary to specify the experimental conditions with some precision; in this work pure DFP was added from a micro-syringe pipette, drop by drop with vigorous swirling of the cell suspension, which consisted of 8 cm³ of blood diluted with 32 cm³ of KCl solution. The value of 0.0091 M, given in figure 1, is thus the concentration the medium would have had if none of the DFP had been decomposed, and none taken up by the cell membranes.

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THE USE OF SPISULA SOLIDISSIMA EGGS IN CELL RESEARCH

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In view of the fact that marine egg material is so important for the cell physiologist, it is unfortunate that this material is not available during the winter months to most of the workers along the Atlantic seaboard. Recently it has been found possible to obtain large numbers of the common surf clam, Spisula solidissima (Dillwyn) from commercial dredgers along the New Jersey coast. (This clam is always referred to by American experimentalists as Mactra solidissima.) Spisula has various advantages. In the first place it can be transported easily without much inquiry and can be kept dry in a cold place for several days without damage to the gametes. (Even at Woods Hole the animals are obtained at a considerable distance from the Marine Biological Laboratory and are carted to the laboratory by truck.) Secondly, Spisula has vast numbers of ripe eggs at various times of the year; this is of great importance to workers interested in biochemical problems. If Spisula were better understood, it might well become a standard source of material for workers along the Atlantic coast. Moreover, at Woods Hole it would serve to conserve the already short supply of sea urchins. Thirdly, Spisula eggs are relatively free of yolk and unpigmented; changes in the cortical layer, the cytoplasm and the large germinal vesicle can be readily followed in great detail with phase contrast microscopy. About 10 minutes after insemination or artificial activation, the breakdown of the germinal vesicle gives the observer insight into the percentage of eggs that have been affected, and perhaps the percentage of cleavage to be expected.

Unfortunately, in the past, workers at Woods Hole have not properly understood how to obtain synchronous devel-

opment from Spisula eggs. In experimental studies of cell division, marine eggs must cleave as nearly simultaneously as possible. With sea urchin eggs it is a simple matter to obtain cleavage at a uniform time. But this is not so easy with Spisula. If the eggs are removed from the ovary and inseminated directly, from 50–100% will be fertilized, and usually many of these will be delayed or inhibited sometime before the first mitotic division. This is apparently due to the presence of inhibitory substances in the ovary. Fortunately, by frequent washings it is possible to obtain practically 100% of fertilization and simultaneous cleavage.

Eggs are best obtained by removing the hinge of a clam, breaking the ovary and forcing the eggs to exude into a beaker of filtered sea-water. The eggs are then permitted to settle to the bottom for a few minutes. The supernate, which contains debris, follicle cells and immature oocytes is then poured off, leaving the heavier and more mature eggs at the bottom. For best results this process must be repeated at least 4 times within the space of a half hour. Dry ovaries and testes can be kept in the refrigerator overnight, and washed eggs can be kept for 4 to 6 hours. Sperm concentrations must be kept quite low; one drop of dry sperm in 10 ml of filtered sea-water makes a convenient stock solution, of which no more than two drops should be added to eggs in 40–50 ml of filtered sea-water. Spisula eggs are quite susceptible to polyspermy at high sperm concentrations. However, extra pronuclei can be easily detected after polar body formation and before the first cleavage. Perhaps the most important consideration in the use of Spisula eggs in their susceptibility to crowding; this situation can be avoided by limiting the number of eggs in the low power microscopic field to 20 or 25.

If, then, Spisula eggs are fertilized with the proper amount of sperm, and are placed sufficiently far apart in the experimental dish, they can be expected to show between 97 and 100% cleavage.

ERYTHROCYTE VOLUME AND UREA IN ELASMOBRANCHS

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TWO FIGURES

It has been generally known since the work of Rodier (1899) and of Smith ('29) that the blood urea concentration of the elasmobranch fishes exerted about one-third of the total measured osmotic pressure of such blood. When Jacobs ('31) showed that the erythrocyte of elasmobranchs was relatively permeable to urea it became possible to predict that the equilibrium volume of these cells was independent of the blood urea content. This paper reports experimental verification of this prediction.

The studies described below arose from a search for isotonic solutions for fish red cells including those of the smooth dogfish *(Mustelis canis)* and the sting ray *(Dasyatis centrura)*. All experiments were performed at a room temperature of 23°C. The experimental finding that a solution containing 19.8 gm of NaCl and 19.2 gm of urea per liter (approximately equivalent osmotically to 0.5 M NaCl) and a solution of 0.34 M NaCl were both isotonic for dogfish red cells indicated a lack of effect of urea on the equilibrium volume of these cells; since the NaCl concentration in the NaCl-urea solution was 0.33 M.

To check this point, dogfish red cells were exposed to a series of solutions in which the NaCl concentration was maintained constant at 0.35 M while the urea concentration was varied from 0 to 1500 mg %. The experiment was performed in the densimeter described by Parpart ('35) which measures volume changes from the first second following the mixing of cells with a solution. The results are shown in figure 1. The volume changes which occurred are primarily due to outward or inward passage of water. In each case the final

volume was the same as the initial volume irrespective of the
urea content. The rate at which equilibrium was approached
was, however, determined by the concentration difference of
urea on the two sides of the cell membrane.

The converse experiment with the red cells of the dogfish
and sting ray was conducted by maintaining the urea con-
centration constant at 1500 mg % and varying the NaCl con-
centration. The results of such an experiment with sting
ray cells is shown in figure 2. The final equilibrium volume
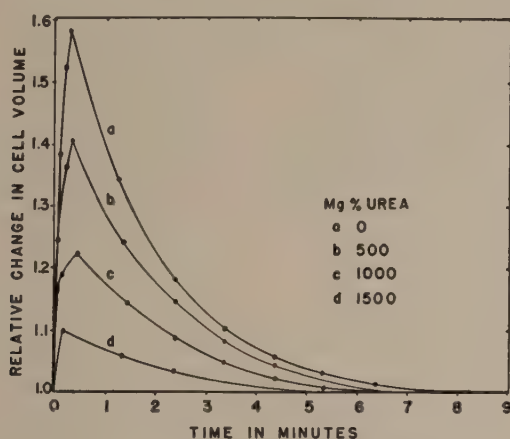
Fig. 1 The effect of alteration in the concentration of urea on the equilib-
rium volume of dogfish red cells in 0.35 M NaCl. The ordinate, relative cell
volume, refers to increases in cell volume compared to the initial volume at one.
The units are arbitrary and were determined from the galvanometer deflection.

of the cells was altered with the concentration of the NaCl
solution. The initial rapid rise or drop in the curves is due
to the entrance or exit of water. The slow drop in each curve,
following the attainment of a temporary peak, is due to the
outward diffusion of urea and the consequent outward pas-
sage of water from the cells until a true equilibrium is reached
with the urea in the external medium. This outward diffusion
of urea indicates that the initial intracellular concentration
of urea was greater than 1500 mg %. The fact that the out-
ward diffusion of urea occurred at the same rate in all salt

concentrations means that water exchanges were primarily involved in the initial volume changes of the cells.

These experiments demonstrate that the urea present in the blood of elasmobranchs studied exerts no measurable in-
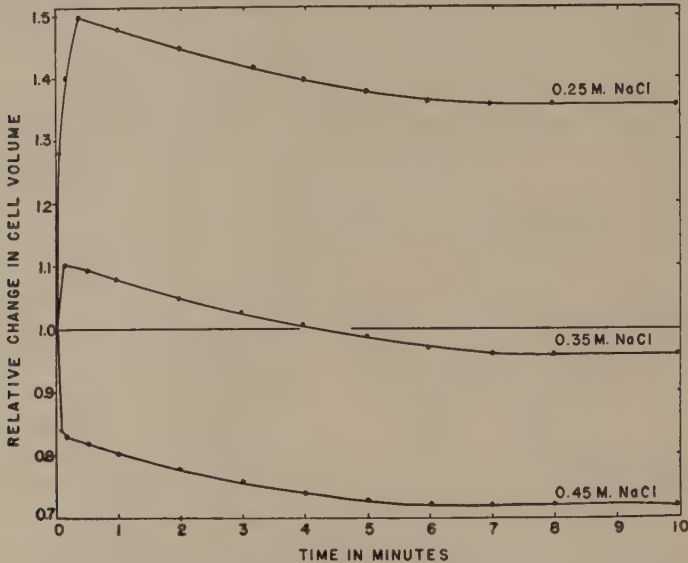
Fig. 2 Sting ray red cells. The effect of varying the NaCl concentration while maintaining the urea concentration constant at 1500 mg %. The ordinate, relative changes in cell volume, refers to increases or decreases in cell volume compared with the initial volume at one. The units are arbitrary and were determined from the galvanometer deflection.

fluence on the equilibrium volume of the red cells but that this volume is determined by the concentration of the salts present.

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